REMARKS

This Reply is responsive to the Office Action dated October 2, 2001. Entry of the foregoing and reconsideration on the merits pursuant to 37 CFR 1.112 is respectfully requested.

The application has been amended as set forth above. In accordance for the new rules for amending applications set forth in 37 CFR 1.121, which took effect on March 1, 2001, a marked up version of the claims showing all amendments is attached hereto as an appendix.

Specifically, claim 1 has been amended for grammatical purposes, and to emphasize the pluripotent nature of the embryonic stem-like cells obtained by the recited method. For instance, the specification makes clear at page 50, line 16 to page 51, line 3, that the embryonic stem-like cells of the invention are obtained out of a nuclear transfer unit, and as such could not be used for germ-line manipulation. New claim 55 further emphasizes the pluripotent nature of the embryonic stem-like cells obtained by the claimed methods by specifying that the cells are obtained from the inner-most portion of the nuclear transfer unit. Support for this claim may be found at page 50, line 21. New claim 56 is directed to an embryonic stem-like cell isolated by the method of claim 55. New claims 57 and 58 replace claim 35, which was canceled above. No new matter was added.

In addition, the specification was amended to include an updated status of priority applications, and to correct an inadvertent grammatical error on page 20. No new matter was added.

Turning now to the Office Action, the declaration submitted with the application was noted to be defective because inventor Lanza neglected to date his signature. Accordingly, a new declaration **is attached hereto.**

The specification was objected to because of the recitation of "karyoplast?????" on page 20. The specification was amended above to delete the question marks at this cite. Therefore, this objection is now moot.

The specification was also objected to because a brief description of the figures was included but no figures were submitted. Applicants have attached hereto the figures (formal photographs) that were inadvertently omitted when the present application was filed. Entry into the application is respectfully requested. Applicants note that the figures were filed with

the priority application, PCT/US99/04608, which was incorporated by reference by way of the transmittal filed along with the present application. Accordingly, no new matter is added.

Claims 27-32 were rejected under 35 U.S.C. §101. According to the Office Action, the claims read on cells that are a human embryo. Applicants respectfully disagree.

The rejected claims were directed to embryonic or stem-like cells made according to the methods of the invention. The term "embryonic or stem-like cells" was adopted to reflect the unique nature of the cells of the invention, i.e., in that they contain a nucleus from one species and the mitochondria from another (see page 18, lines 6-13). In order to clarify that the claims are directed to cross-species, nuclear transfer-derived cells, the claims have been amended to delete the "or" from the phrase "embryonic or stem-like cells." In addition, the preamble of claim 1 has been amended to define the term "embryonic stem-like cells," and to clarify the cross-species nature of cells to maintain the intended spirit of the invention.

The fact that the embryonic stem-like cells produced by the claimed methods comprise a nucleus derived from an adult differentiated cell and mitochondria from an oocyte of species other than the adult differentiated cell renders such cells different from any other embryonic cell or embryo known in the art or in nature. According to the U.S. Supreme Court in Diamond v. Chakrabarty, 447 U.S. 303, 206 USPQ 193 (1980), the test for patentability under 35 U.S.C. §101 is whether the living matter is the result of human intervention. See also MPEP 2105. The point was also made in Chakrabarty that "Congress thus recognized that the relevant distinction was not between living and inanimate things, but between products of nature, whether living or not, and human made interventions." Applicants respectfully submit that an embryonic stem-like cell wherein the nuclear and mitochondrial DNAs are cross-species with regard to one another could only be a product of human intervention, and therefore, such cells satisfy the test for §101 patentability set forth in Chakrabarty.

Furthermore, unlike an actual human embryo, the embryonic stem-like cells of the present invention cannot develop into a human being because they are not totipotent. Indeed, it is clear from the specification at the paragraph bridging pages 50-51, for instance, that the nuclear transfer units of the present invention will be used to isolate pluripotent cells, not as a totipotent entity. Totipotency is only realized upon implantation of the nuclear transfer unit into a surrogate female, which is never suggested in the present application.

The attached article by Iwasaki and colleagues supports applicants' arguments (Biol. Repro., 2000, 62: 470-75). The objective of this study was to derive offspring from bovine ES-like cells isolated from the inner cell mass of an in vitro produced embryo. However, to generate offspring, these ES-like cells had to be aggregated with tetraploid embryos, thereby forming a chimeric embryo. The ES-like cells alone could not have been used to derive offspring, because ES-like cells are taken out of the inner cell mass of an embryo or nuclear transfer unit and as such only constitute part of a complete embryo. Specifically, they do not have the capability to generate an embryo when implanted into a surrogate female because the cells are taken from a nuclear transfer unit that has differentiated past the stage where the trophectoderm (extra-embryonic part of the ectoderm of mammalian embryos at the blastocyst stage) forms.

Nevertheless, although ES-like cells cannot be implanted alone into a surrogate female to generate an embryo, fetus, or offspring, ES-like cells are pluripotent in that they may differentiate into all the cells of the body. Indeed, while the trophectoderm gives rise to extra-embryonic tissues involved in implantation and maintenance of the fetus during development, the inner cell mass gives rise to the fetus itself. In order to emphasize the pluripotent nature of the cells (and distinguish them from totipotent entities), claim 1 has been amended above to clarify that the claimed embryonic stem-like cells are isolated from a disassociated nuclear transfer unit. More specifically, as recited in new claim 55, the cells are isolated from the "inner-most" portion of the nuclear transfer unit as discussed on page 50, line 21, of the specification.

Thus, while pluripotent cells may differentiate into all cell types of the body, they do not have the capability to generate an embryo when implanted into a surrogate female because the cells are taken from a nuclear transfer unit that has differentiated past the stage where the trophectoderm forms. Further, in view of the amendments to claim 1 above and in view of new claim 55, it should now be clear that the cells of the invention are pluripotent rather than totipotent. Furthermore, the cells of the invention are distinguished from any cell found in nature in that the cells contain a nucleus and mitochondria that are cross-species with regard to each other. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. §101 is respectfully requested.

Next, claims 1-54 were rejected under 35 U.S.C. §112, first paragraph for alleged lack of enablement. Essentially, it is the Examiner's opinion that, although Example 1 of the specification is acknowledged to result in the production of a nuclear transfer unit (16-400 cell stage) that appears to be ES-like, Applicants have allegedly failed to demonstrate that the ES-like cells are totipotent or that they function as stem cells capable of differentiating into other multilineage cell types. The Examiner therefore concludes that Applicants have failed to enable the production of embryonic or stem-like cells as recited in the claims. The Examiner further alleges that, because it is unclear whether the resultant ES-like cells differentiate into other cell types useful for transplantation, it is unknown how the skilled artisan would be able "to use" the claimed embryonic or stem-like cells in a manner which is consistent with the specification (page 6 of the Official Action).

The Examiner also raises the point that it is allegedly inappropriate to rely on the prior art with respect to any other species with regard to ES cell differentiation techniques because results are species dependent, and one would not reasonably expect to induce cell differentiation in other cell lineages using techniques available for mouse ES cells (pages 6-7 of the Office Action). The Examiner also questions the contribution of the oocyte cytoplasm or mitochondria in the cross-species ES-like cells of the invention, and relies on a reference by Dominko et al. as suggesting that the success of cross-species nuclear transfer cannot be judged until somatic cell/recipient cytoplasm compatibilities are examined (pages 7-8). The Examiner makes specific opposition to the terminology "embryonic or stem-like cells" to the extent that it reads on common embryonic stem cells (page 9). In this regard, the Examiner notes that true human ES cells have the potential to develop into a human being, and suggests that Applicants choose different claim terminology if this is not an intended use of the cells (page 9).

Finally, with regard to claims 36-54, the Examiner notes that the specification fails to teach gene modification of any differentiated cell, and fails to teach the use of gene-modified cells as a starting point for nuclear transfer (page 10). Applicants respectfully traverse all these grounds for the rejection, and request reconsideration with regard to each claim taken individually in light of the following remarks and the amendments entered above.

First, Applicants again note the amendments submitted above. Because it appears that one of the Examiner's main concerns is the terminology "embryonic or stem-like cells,"

Applicants have clarified this phrase in the preamble of Claim 1 by emphasizing that such cells comprise a nucleus derived from an adult differentiated cell and mitochondria from an oocyte of a species other than said adult differentiated cell. Thus, the cells produced by the claimed method are different from embryonic stem cells as they are commonly known in the art, in that the cells contain nuclear DNA and mitochondrial DNA respectively derived from different species.

Nevertheless, the fact that the ES-like cells of the present invention contain xenogeneic mitochondria does not mean that such cells are not capable of differentiating into other cell types, or even differentiating into a mammal or serving as part of a chimeric mammal (if a suitable vehicle for such development was provided). That such cross-species differentiation and development is possible is evidenced by recent data gathered by the present inventors which shows that it is possible to use nuclear transfer from a somatic cell from a gaur into an enucleated oocyte of a bovine to produce a cloned gaur having bovine mitochondria. A copy of a recent publication detailing these results is attached for the Examiner's review, and Applicants would be amenable to submitting this data in the form of a declaration if the Examiner indicates that this would be helpful.

While the production of the cloned gaur is evidence that cross-species nuclear transfer may be used to produce embryonic or stem-like cells which differentiate and develop into a mammal, Applicants again emphasize that their intention in using human cells as nuclear transfer donors is not to produce adult cloned humans. Rather, cross-species nuclear transfer can be valuable in the production of human ES-like cells that may be used to produce differentiated cells and tissues for the purpose of transplantation. While Applicants acknowledge that the instant specification does not disclose the actual production of human differentiated tissues, it is entirely reasonable to expect that such results are readily achievable given the production of a cloned gaur using cross-species transplantation, and the novel showing by Applicants that human cross-species nuclear transfer into a bovine generates a unit with ES cell-like morphology. It also follows that such tissues would be ideally suited for transplantation and cell therapies given that the tissues may be designed using a cell or nucleus from the patient in need of such transplantation or therapy.

Furthermore, the generation of differentiated cells for transplantation is not the only utility for the nuclear transfer units generated by the claimed methods. For instance, on page

2 of the specification, Applicants note that embryonic stem cells provide an *in vitro* model for differentiation, and as such can be used in the study of genes which are involved in the regulation of early development. Human cross-species ES-like cells in particular can be used to identify important human regulatory genes, and would be highly useful in this regard even if they were not used for transplantation purposes. Indeed, the fact that Applicants have found that cross-species nuclear transfer of a human cell into a bovine cell generates an activated nuclear transfer unit capable of division is an enormously exciting and important finding. For example, such cells provide a model for deciphering the role of the mitochondrial genome in mammalian development and cellular function.

Thus, Applicants believe that the activated cross-species nuclear transfer units claimed in the present invention are fully enabled by the specification, and moreover have significant utility even if they are not used to produce differentiated cells and tissues for transplantation. However, Applicants respectfully emphasize that this does not mean that such activated nuclear transfer units could not be used to produce embryonic stem-like cells capable of being used for this purpose. Rather, Applicants believe the gaur data attached hereto demonstrates that cross-species nuclear transfer may be used to generate differentiated cells and tissues.

Finally, with regard to gene-modified differentiated cells, such cells have been commonly produced in the art for some time so it cannot be argued that it would require undue experimentation to transfect a differentiated cell with a gene of interest. Indeed, the present invention provides an advantage over previous nuclear transfer methods by using somatic cells; prior art methods typically employed embryonic cells which are more difficult to carry in culture and hence more difficult to modify with a gene knock-in or knock-out. Yet, researchers have been genetically modifying ES cells for years. Thus, there is no reason to believe that the differentiated donor cells used in the present invention could not be genetically modified as described in the present disclosure in order to affect the claimed methods. Furthermore, there would be no reason to believe absent some evidence to the contrary that a donor cell containing a heterologous gene could not just as readily be used as a donor cell for nuclear transfer as described in the present disclosure.

In support of the above, attached hereto is a publication by Cibelli et al. (Nature Biotechnol., 1998, 16:642-46), showing the production of transgenic ES-like cells from fetal

fibroblasts transfected with a CMV-ß-galactosidase-neomycin (ß-Geo) heterologous cassette. As described in the reference on the first page, second column, the transgenic fibroblast cells were fused with enucleated oocytes to produce blastocyst stage nuclear transplant embryos. Furthermore, as shown in Figure 2 and discussed on the second page of the reference, transgenic ES-like cells expressing a heterologous gene could be derived by either transfecting donor cells prior to nuclear transfer (right column of Figure 2), or by transfecting ES-like cells isolated from the inner cell mass of a blastocyst stage embryo. Moreover, both types of ES-like cells were used to produce chimeric calves, wherein expression of the transgene could be detected in cells derived from all three germ layers.

Thus, the attached publication by Cibelli et al shows (1) that transgenic ES-like cells may be made using known techniques for transfection at any stage in the claimed method, i.e., by transfecting a heterologous gene before or after nuclear transfer of the donor cell or cell nucleus, and (2) that transgenic ES-like cells have the ability to differentiate into all different cells of the body. Although the studies reported by Cibelli used cells of the same species (bovine) as donor and recipient for nuclear transfer, there is no reason to believe that the same could not be done with cross-species nuclear, particularly given the showing above that cross-species nuclear transfer results in the birth of a cloned gaur.

Thus, Applicants believe that the activated cross-species nuclear transfer units and embryonic stem-like cells are fully enabled by the specification, and moreover have significant utility even if they are not used to produce differentiated cells and tissues for transplantation, i.e., as models for *in vitro* differentiation. However, Applicants respectfully emphasize that this does not mean that such activated nuclear transfer units could not be used to produce embryonic stem-like cells capable of being used to produce differentiated cells and tissues. Rather, Applicants believe the gaur data previously submitted demonstrates that cross-species nuclear transfer may be used to generate differentiated cells and tissues. Furthermore, the successful birth of a cloned gaur using cross-species nuclear transfer suggests that cytoplasmic and mitochondrial differences between donor and recipient cells in nuclear transfer are not surmountable. With the claimed methods for optimizing nuclear transfer by transferring compatible donor cytoplasm and cytoplasm components such as mitchondria, cross-species nuclear transfer should readily be extended to any combination of donor and recipient species.

For all the above reasons, withdrawal of the enablement rejection under 35 U.S.C. §112, first paragraph is respectfully requested.

Claims 1-54 were rejected under the second paragraph of 35 U.S.C. §112 for alleged indefiniteness. The rejections will be addressed in the order presented for the convenience of the Examiner.

Claims 1, 24-32 and 38 were rejected for the recitation of "embryonic or stem-like cells." According to the Office Action, it is unclear whether this phrase is meant to encompass embryonic stem cells, stem cell progenitors or precursor cells or both. In addition, the Examiner questions the use of the phrase "stem cell-like" with respect to the contribution of bovine oocyte mitochondria, noting that if the contribution of the bovine mitochondria has absolutely no effect on the resulting cells, then the cells should be termed embryonic stem cells and the claims should be amended accordingly. Applicants respectfully disagree.

The claims have been amended to replace the phrase "embryonic or stem-like cells" with "embryonic stem cells," and to include a definition of the cell in the preamble of claim 1. This amendment should resolve the rejection with regard to the scope of the claimed phrase. However, Applicants respectfully submit that the presence of xenogeneic mitochondria renders such cells physically distinct from other cells as to warrant the phraseology. Although the presence of xenogeneic mitochondria does not deter the differentiation of ES-like cells as demonstrated by the birth of the cloned gaur, the fact that the cells possess xenogeneic mitochondria makes them physically distinct from other cells of mammals of the same species as the donor, both on an intracellular and extracellular level.

For instance, according to the enclosed article by Bruce Loveland and others, cells display peptides derived from mitochondrially encoded proteins, and such peptides can serve as histocompatibility antigens. Loveland et al., 1990, Maternally transmitted histocompatibility antigen of mice: A hydrophobic peptide of a mitochondrially encoded protein, Cell 60: 971-80 (see the abstract). According to Wheeler et al., also attached, the mitochondrial genome encodes thirteen proteins, and defects in the mitochondrial genome "play an important and increasingly apparent role in human disease." Wheeler et al., 1997, Modification of the mouse mitochondrial genome by insertion of an exogenous gene," Gene 198: 203-209 (see p. 203, col. 1).

Thus, embryonic stem-like cells derived by cross-species nuclear transfer cannot be compared to stem cells derived from the original donor species, because embryonic stem-like cells contain different mitochondria, which in turn encode different proteins. Moreover, peptides from such mitochondrial proteins are displayed on the surface of differentiated cells, rendering such cells distinctly different both on the inside and outside surface of the cells. Embryonic stem-like cells that contain xenogeneic mitochondria are therefore structurally distinct from embryonic stem cells known in the art, and the different terminology to stress this distinction is therefore warranted.

Claim 1 was rejected because it is unclear how the recitation of "("compatible cytoplasm")" relates to the rest of the claim. As the phrase "compatible cytoplasm" was deleted by way of amendment above, this rejection is now moot. Applicants believe that the meaning of the claim is clear in the absence of the parenthetical.

Likewise, Claim [2] was rejected because it is unclear how the recitation of "("compatible mitochondria")" relates to the rest of the claim. As the phrase "compatible mitochondria" was deleted by way of amendment above, this rejection is now moot. Applicants believe that the meaning of the claim is clear in the absence of the parenthetical.

Claim 35 was rejected because there was alleged to be insufficient antecedent basis for the limitation "which contain and express an inserted gene." Without necessarily agreeing with the rejection, applicants have canceled claim 35 in favor of new claims 57 and 58, which are dependent on claim 36 which in turn was amended to provide the appropriate antecedent basis. Therefore, the rejection should now be withdrawn.

Claim 36 was also rejected because there was alleged to be insufficient antecedent basis for the limitation "wherein a desired gene is inserted, removed or modified." Claim 36 was amended above to rephrase the limitation as a further method step, thereby negating the need for antecedent basis support in the base claim. Reconsideration and withdrawal of the rejection is respectfully requested.

Claim 50 was rejected because of the recitation of "a DNA that encodes a detectable marker, the expression of which is linked to a particular cyclin." Applicants believe that the meaning of this phrase is clear in view of the disclosure at page 49, lines 5-14, discussing constructs comprising a cyclin DNA that is operably linked to a regulatory sequence together with a detectable marker, such as GFP (green fluorescence protein). Nevertheless, claim 50

was amended above to clarify that expression of the marker is operably linked to expression of the cyclin. Withdrawal of the rejection is respectfully requested.

Turning now to the prior art rejections, claim 27 was rejected under 35 U.S.C. §102(b) as being allegedly anticipated by Bradley et al. (Biotechnol. 1992), who teach mouse embryonic stem cells. According to the Office Action, the rejection was made because the phrase "embryonic or stem-like cells" was interpreted as embryonic stem cells. Applicants respectfully submit that the rejection based on Bradley should now be moot in view of the discussion submitted above in response to the rejection under §112,second paragraph, where it was shown that the embryonic stem-like cells may be structurally distinguished from embryonic stem cells of the prior art. Reconsideration and withdrawal of the rejection is respectfully requested.

Likewise, Claims 27-32 were rejected under 35 U.S.C. §102(a) as being allegedly anticipated by Granerus et al. Again, because the Examiner was of the opinion that the phrase "embryonic or stem-like cells" encompassed the embryonic stem cells of the prior art, the human embryonic stem cell line, Tera 2, of Granerus was alleged to anticipate the embryonic or stem-like cells of the present invention. However, Applicants respectfully submit that it is now clear that the embryonic stem-like cells of the present invention contain xenogeneic mitochondria in view of the amendments presented above. Moreover, no prior art reference describes such cells, including Granerus. Withdrawal of the rejection in view of the above amendments and remarks is respectfully requested.

Likewise, Claims 27-34 and 50-54 were rejected under 35 U.S.C. §102(e) as being allegedly anticipated by Tsukamoto et al. Again, because the Examiner was of the opinion that the phrase "embryonic or stem-like cells" encompassed the embryonic stem cells of the prior art, the human hematopoietic stem cells of Tsukamoto were alleged to anticipate the embryonic or stem-like cells of the present invention. However, Applicants respectfully submit that it is now clear that the embryonic stem-like cells of the present invention contain xenogeneic mitochondria in view of the amendments presented above. Moreover, no prior art reference describes such cells, including Tsukamoto. Withdrawal of the rejection in view of the above amendments is respectfully requested.

Likewise, Claims 27-34 and 50-54 were rejected under 35 U.S.C. §102(b) as being allegedly anticipated by Yamane et al. Again, because the Examiner was of the opinion that

the phrase "embryonic or stem-like cells" encompass the stem cells of the prior art, the human epithelial and endothelial stem cells of Yamane were alleged to anticipate the embryonic stem-like cells of the present invention. However, Applicants respectfully submit that it is now clear that the embryonic stem-like cells of the present invention contain xenogeneic mitochondria in view of the amendments presented above. Moreover, no prior art reference describes such cells, including Yamane. Withdrawal of the rejection in view of the above amendments is respectfully requested.

Claims 27-36 and 50-54 were rejected under 35 U.S.C. §103(a) as being allegedly unpatentable over Tsukamoto. Note that Claim 35 has been canceled in favor of new Claims 57-58 to provide antecedent basis for the inserted gene. Nevertheless, it is the Examiner's opinion that it would have been obvious to modify differentiated human cells produced from stem cells as described by Tsukamoto to arrive at the genetically modified cells as claimed in the present claims. However, the embryonic stem cells of Tsukamoto cannot reasonably be interpreted as anticipating or rendering obvious the embryonic stem-like cells of the present invention because the cells described in the present invention have xenogeneic mitochondria. Therefore, withdrawal of this rejection is respectfully requested.

Clams 1-34 and 50-54 were rejected under 35 U.S.C. §103(a) as being allegedly unpatentable over Wolfe et al., Collas et al. and Westhusian. Essentially, it is the Examiner's opinion that, although Wolfe et al. fails to teach cross-species nuclear transfer of human or mammalian differentiated nuclei (teaching only cross-species nuclear transfer using bovine nuclei from embryos), it would have been obvious to use differentiated cells in view of the teaching in Collas et al. that a variety of differentiated mammalian cell types might be used for nuclear transfer. Applicants respectfully traverse, however, on the basis that Collas et al. makes no mention that such differentiated cells might be used in cross-species nuclear transfer, and Westhusin adds nothing to make up for this deficiency. It would not have been reasonable to expect at the time the present invention was made that an adult differentiated somatic cell could be reprogrammed by an enucleated oocyte of an entirely different species, let alone a species as evolutionarily diverse as a bovine is to a human nuclear donor.

Furthermore, it would not have been reasonable to expect based on the combined teachings of Wolfe et al., Collas et al. and Westhusin that cross-species nuclear transfer using an adult differentiated cell could be used to generate embryonic-like stem cells that could then

be used to produce differentiated cells, tissues for transplantation, and even live mammals. Indeed, as the Examiner even acknowledges by reference to Dominko et al. (page 7 of the Office Action), successful reprogramming is only satisfactorily proven when one can show a pregnancy carried to term. With the eminent birth of the cloned gaur using cross-species nuclear transfer, and the detailed characterization of the fetal development of the cloned gaur, Applicants are the first to actually demonstrate that cross-species nuclear transfer can be used to generate cloned tissues and mammals from an adult differentiated cell, wherein such mammals are shown to have mitochondria derived from the original recipient oocyte.

The Federal Circuit has recognized that one way for a patent applicant to rebut a *prima facie* case of obviousness is to make a showing of "unexpected results," i.e., to show that the claimed invention exhibits some superior property or advantage that a person of ordinary skill in the relevant art would have found surprising or unexpected. The basic principle behind this rule is straightforward- that which would have been surprising to a person of ordinary skill in a particular art would not have been obvious. The principle applies most often to the less predictable fields, such as chemistry, where minor changes in a product or process may yield substantially different results. In re Soni, 34 USPQ2d 1684, 1687 (Fed. Cir. 1995).

By the Examiner's own discussion in the enablement rejection, and given the statements in Dominko et al concerning the lack of predictability expected in producing cross-species nuclear transfer derived differentiated cells and tissues, one of ordinary skill in the art would not have expected at the time the invention the present invention was made that cross-species nuclear transfer could be used to generate fully developed mammals. The results achieved with the cross-species cloned gaur would therefore have been highly unexpected, and certainly would not have been obvious in view of the combined disclosures of Wolfe, Collas and Westhusin. Indeed, neither Collas nor Westhusin concern cross-species nuclear transfer.

In view of the fact that Wolfe et al. fails to teach cross-species nuclear transfer from an adult differentiated cell, and given that the teachings in Collas et al. regarding the use of differentiated cells can only reasonably be applied to same-species nuclear transfer, and seeing as Westhusin fails to make up for the deficiencies of Wolfe and Collas,

reconsideration and withdrawal of the rejection under §103(a) based on Wolfe, Collas and Westhusin is respectfully requested.

All issues raised by the Office Action dated October 2, 2001, have been addressed in this Reply. Accordingly, a Notice of Allowance is next in order. If the Examiner has any further questions or issues to raise regarding the subject application, it is respectfully requested that the undersigned be contacted so that such issues may be addressed expeditiously.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached Appendix is captioned **"Version with markings to show changes made"**.

Respectfully submitted,

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APPENDIX: VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Page 20, please delete the paragraph starting at line 11 and replace it with the following paragraph:

--The preferred embodiment of the invention comprises the production of non-human primate or human embryonic or stem-like cells by transplantation of the nucleus of a donor human cell or a human cell into an enucleated human, primate, or non-primate animal oocyte, e.g., an ungulate oocyte, and in a preferred embodiment, a bovine enucleated oocyte. Preferably, the enucleated oocyte will also be injected with human cytoplasm (e.g., from at least one immature or mature oocyte or blastomere), or fused with a karyoplast[?????] (enucleated human oocyte or blastomere, or that of a higher primate) and/or human mitochondrial DNA.--

In the Claims:

1. (Amended) A method of producing embryonic [or] stem-like cells, wherein said cells comprise a nucleus derived from an adult differentiated cell and mitochondria from an oocyte of a species other than said adult differentiated cell, comprising the following steps:

(i) inserting a donor differentiated human or mammalian cell or cell nucleus into a recipient animal oocyte, wherein such oocyte is derived from a different animal species than the human or mammalian cell under conditions suitable for the formation of a nuclear transfer (NT) unit, wherein the endobenous oocyte nucleus is removed or inactivated before, concurrent, or after introduction of donor cell or nucleus;

(ii) activating the resultant nuclear transfer unit;

(iii) additionally inserting into said oocyte cytoplasm derived from an oocyte or blastomere of the same species as the donor cell or nucleus [("compatible cytoplasm")];

(iv) culturing said activated nuclear transfer unit until greater than the 2-cell developmental stage; [and

(v) culturing cells obtained from said cultured NT units to obtain embryonic or stem-like cells.]

(v) disassociating said activated nuclear transfer unit; and

(vi) isolating cells from said disassociated nuclear transfer unit to obtain embryonic stem-like cells.

2. (Amended) The method of claim 1, which further includes introducing the mitochondrial DNA of the same species as the donor cell or nucleus into the recipient oocyte [("compatible mitochondria")].

27. (Amended) Human embryonic [or] stem-like cells obtained according to the method of Claim 1.

28. (Amended) Human embryonic [or] stem-like cells obtained according to the method of Claim 11.

29. (Amended) Human embryonic [or] stem-like cells obtained according to the method of Claim 12.

30. (Amended) Human embryonic [or] stem-like cells obtained according to the method of Claim 13.

31. (Amended) Human embryonic [or] stem-like cells obtained according to the method of Claim 14.

32. (Amended) Human embryonic [or] stem-like cells obtained according to the method of Claim 15.

36. (Amended) The method of Claim 1, [wherein] further comprising a step whereby a desired gene is inserted, removed or modified in said embryonic [or] stem-like cells.

50. (Amended) A mammalian somatic cell that expresses a DNA that encodes a detectable marker, the expression of which is operably linked to the expression of a particular cyclin.

Biology of Reproduction 62, 470-475 (2000)

Articles

Production of Live Calves Derived from Embryonic Stem-Like Cells Aggregated with Tetraploid Embryos[1]

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▷ ABSTRACT

To date, cloned farm animals have been produced by nuclear transfer from embryonic, fetal, and adult cell types. However, mice completely derived from embryonic stem (ES) cells have been produced by aggregation with tetraploid embryos. The objective of the present study was to generate offspring completely derived from bovine ES-like cells. ES-like cells isolated from the inner cell mass of in vitro-produced embryos were aggregated with tetraploid bovine embryos generated by electrofusion at the 2-cell stage. A total of 77 embryo aggregates produced by coculture of two 8-cell-stage tetraploid embryos and a clump of ES-like cells were cultured in vitro. Twenty-eight of the aggregates developed to the blastocyst stage, and 12 of these were transferred to recipient cows. Six calves representing 2 singletons and 2 sets of twins were produced from the transfer of the chimeric embryos. Microsatellite analysis for the 6 calves demonstrated that one calf was chimeric in the hair roots and the another was chimeric in the liver. However, unfortunately, both of these calves died shortly after birth. Two of the placentae from the remaining pregnancies were also chimeric. These results indicate that the bovine ES-like cells used in these studies were able to contribute to development.

▷ INTRODUCTION

Since Willadsen [1] reported that live lambs could be produced by

▫ INTRODUCTION
▽ MATERIALS AND METHODS
▽ RESULTS
▽ DISCUSSION
▽ REFERENCES

the transfer of genetic material from blastomeres at the 8-cell stage into enucleated oocytes, cells from developmentally more-advanced embryo stages have been used as donors of genetic material, including cells of the inner cell mass (ICM) in both cattle [2–4] and sheep [5]. More recently Campbell et al. [6] succeeded in producing cloned sheep using a cultured differentiated cell population isolated from the embryonic disc of Day 9 ovine embryos. Since this report, live offspring have been produced by nuclear transfer using donor nuclei from fetal and adult cells in a range of species including sheep [7], cattle [8], mice [9], and goats [10]. These breakthroughs have proven that the nuclei from somatic cells can be reprogrammed by a combination of cell starvation and the introduction of cells into enucleated metaphase II oocytes. In animals produced by nuclear transfer, the recipient cytoplasts are obtained from different donors, and studies have shown that in the resultant animals, the mitochondria are derived almost exclusively from the donor oocyte. Therefore, in a group of cloned animals, the mitochondrial DNA is not from the same source as the donor nucleus.

In contrast to this, Nagy et al. [11,12] have reported the birth of mice completely derived from embryonic stem (ES) cells. In this case, the mitochondria would also be derived from the same cell as the nuclear genetic material. The technique used in these experiments (darning needle method), which involves the aggregation of ES cells with tetraploid embryos, has a number of advantages: technically, it does not require high skill levels or expensive equipment [13,14]; scientifically, the resultant animals could be classified as clones rather than genomic copies as produced by nuclear transfer. To use this approach in species other than the mouse, it is necessary to establish an ES or ES-like cell line and to prepare tetraploid embryos. In cattle, previous studies have reported the production of chimeric calves when ICM cells were either injected into the blastocoelic cavity [15] or aggregated with morula-stage embryos [16]. In addition, Saito et al. [17] succeeded in establishing a bovine ES-like cell line and producing a chimeric calf by aggregation with morula-stage embryos. More recently, Cibelli et al. [18] reported that transgenic bovine chimeras were produced from transgenic ES-like cells and transgenic somatic cell-derived stem-like cells microinjected into Day 3 embryos. This study also indicated that ES-like cells could be produced from somatic cell genomic material by nuclear transfer.

because ES cells cannot develop into animal alone!

In a previous paper [19], we reported that some bovine embryos produced by aggregation of 2 putative tetraploid embryos with either a clump of ICM cells or mammary epithelial cells developed to the blastocyst stage. In addition, the ICMs of these aggregates were derived partly or completely from the donor cells [19]. We report here that the chimeric calves can be produced from the aggregation of ES-like cells with tetraploid embryos.

▷ MATERIALS AND METHODS

▵ TOP
▵ ABSTRACT
▵ INTRODUCTION
▫ MATERIALS AND METHODS
▽ RESULTS

Preparation of 2-Cell Bovine Embryos

Two-cell-stage bovine embryos used to produce tetraploid embryos were prepared in vitro as described previously [20]. Briefly, F1 (Japanese Black Cattle x Holstein breed) oocytes were matured in

▽ DISCUSSION
▽ REFERENCES

vitro in tissue culture medium (TCM) 199 (Gibco BRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS) and then fertilized with frozen-thawed sperm of Japanese Black Cattle. Fertilized oocytes were cultured in microdrops of CR1aa medium supplemented with 5% FBS under mineral oil (Squibb, Princeton, NJ) at 38.5°C in a humidified gaseous atmosphere of 3% CO_2 in air. Embryos at the 2-cell stage were obtained at 28 to 30 h post insemination.

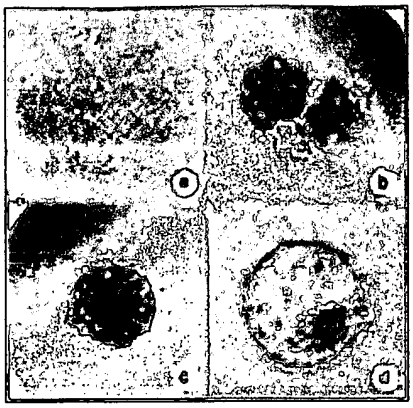
Production of Tetraploid Embryos

Electrofusion was carried out as described by Iwasaki et al. [21], with a minor modification in the fusion medium. Briefly, 7–10 2-cell-stage embryos were washed in a solution containing 0.29 M D(−)-mannitol (Wako, Osaka, Japan), 0.1 mM magnesium sulfate, 0.05 mM calcium chloride, and 0.05% BSA. The washed embryos were then placed in the mannitol solution between the 2 platinum electrodes (1-mm gap) of the fusion chamber (BTX 450-1, San Diego, CA). Cell fusion was induced by application of 2 DC pulses of 1.0 kV/cm for 25 μsec generated with an electric cell fusion processor (SSH-10, Shimadzu, Kyoto, Japan). The treated embryos were washed in CR1aa supplemented with 5% FBS and subsequently transferred into a drop of culture medium with cumulus cells. Embryos that fused within 1 h were later used as tetraploid embryos for aggregation with ES-like cells.

Culture of ES-like Cells and Preparation for Aggregation

An ES-like cell line designated as 137-cells was established from Day 9 blastocysts (obtained from oocytes collected from Holstein cows at a slaughterhouse) subjected to in vitro maturation, in vitro fertilization, and in vivo culture in a sheep oviduct [22]. ICMs were mechanically isolated and plated onto human fetal lung fibroblasts (MRC-5; American Type Culture Collection, Rockville, MD). Then the ICMs were mechanically subcultured several times every 5–7 days in a culture medium consisting of a mixture of Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) and TCM 199 (1:1), 10% FBS, 2 mM L-glutamine, 1 mM minimum essential medium Eagle (MEM) nonessential amino acid solution (Gibco BRL), and 5 x 10^2 units/ml of ESGRO (Gibco BRL). As shown in Figure 1a, 137-cells were cultured from passage 8 in our laboratory with the same culture medium. The 137-cells were cultured in the absence of a feeder layer at 37°C, in a humidified gaseous atmosphere of 5% CO_2 in air, and were trypsinized at 100% confluence to obtain clumps of 10–15 cells for the aggregation. The dish containing the 137-cells was first rinsed with PBS(−), and then trypsin solution was added. The trypsin solution was immediately removed, and the cells were incubated for 30 sec. After the culture medium was added, some clumps of the 137-cells were picked up with a mouth-controlled pipette under a dissecting microscope and washed in CR1aa medium supplemented with 5% FBS.

FIG. 1. Morphology of bovine ES-like cells (137-cells) and development of the aggregates of 2 tetraploid embryos with a clump of 137-cells. (a) The 137-cells. (b) Position of 2 compacting tetraploid embryos with loose clump of 137-cells to prepare the aggregates. (c) Compacting morula embryos at 2 days after aggregation. (d) Blastocyst at 4 days after aggregation



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Aggregation of the 137-Cells with Bovine Tetraploid Embryos

The 137-cells were aggregated with tetraploid embryos as described previously [19]. A small depression was made, using a darning needle (Clover 55–042, Osaka, Japan), in each 15-μl droplet of CR1aa supplemented with 5% FBS. Putative tetraploid embryos were treated with 0.5% Pronase solution (Pronase E; Kaken Kagaku, Chiba, Japan) to remove zonae pellucidae. After the treated embryos were washed in CR1aa-supplemented 5% FBS, 2 zona-free tetraploid embryos at the same developmental stage were put in the depression. One clump of 137-cells washed in CR1aa supplemented with 5% FBS was then put into the depression to form a triangle with the 2 zona-free tetraploid embryos (Fig. 1b). The aggregates were then cultured at 38.5°C in a humidified gaseous atmosphere of 3% CO_2 in air until the blastocyst stage.

Embryo Transfer to Recipient Cows

The aggregates that developed to blastocysts were transferred to recipient cows at 7 or 8 days after estrus. Embryos were transferred to recipients in a number of ways: 1) a single blastocyst to each uterine horn, 2) a single blastocyst to one uterine horn, or 3) transfer of 2 blastocysts to one uterine horn (see Table 2). Pregnancy was detected by ultrasound examination 28 to 31 days after the embryo transfer.

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TABLE 2. Embryo transfer and delivery of calves from aggregates of putative tetraploid embryos and the 137-cells

Chromosome Analysis of Lymphocytes from Calves

Heparinized blood was centrifuged at 2500 rpm for 10 min, and the lymphocyte layer was used for culture in TCM 199 supplemented with 10% FBS and phytohemagglutinin-M (2 μg/ml) for 72 h at 37° C. Subsequently, lymphocyte cultures were treated with vinblastine (0.4 μg/ml) for 5 h and then treated with hypotonic solution (0.9% trisodium citrate) for 8 min. The lymphocytes were fixed in a solution of acetic acid and methanol (1:3) overnight and then mounted on clean glass slides. Dried preparations

were stained with 4% Giemsa solution for 3.5 min, and chromosome number and compositions were analyzed under a microscope with a magnification of 1000x.

Microsatellite Analysis of Genomic DNA

Genomic DNA was extracted from the donor 137-cells, hair roots, liver, placenta, and testes. Microsatellite analysis was carried out using 12 primer sets designated BM2113, BM1824, SPS115, ETH3, ETH225, ETH10, TGLA227, TGLA126, TGLA122, MGTG4B, TGLA53, and SPS113. Polymerase chain reaction (PCR) was carried out under the following conditions: For BM2113, BM1824, and SPS115—93°C for 5 min for first denaturing, 33 cycles of denaturing, annealing, and extending (93°C for 1 min for denaturing, 55°C for 1 min for annealing, and 72°C for 1 min for extending), and 72°C for 10 min for final extension; for ETH3, ETH225, and ETH10—93°C for 5 min for first denaturing, 33 cycles of denaturing, annealing, and extending (93°C for 1 min for denaturing, 68°C for 1 min for annealing, and 72°C for 1 min for extending), and 72°C for 10 min for final extension; for TGLA227, TGLA126, TGLA122, MGTG4B, TGLA53, and SPS113—93°C for 5 min for first denaturing, 29 cycles of denaturing, annealing, and extending (93°C for 1 min for denaturing, 68°C for 1 min for annealing, and 72°C for 1 min for extending), and 72°C for 10 min for the final extension.

▷ RESULTS

△ TOP
△ ABSTRACT
△ INTRODUCTION
△ MATERIALS AND METHODS
▫ RESULTS
▽ DISCUSSION
▽ REFERENCES

Bovine ES-like cells (137-cells) were cultured on gelatinized culture dishes without feeder layers to form a confluent monolayer. The 137-cells proliferated very slowly, requiring 7–8 days for a single population doubling. The morphology of the 137-cells is shown in Figure 1a. Unlike murine ES cells, the 137-cells formed a monolayer. The 137-cells were easily trypsinized to form a single cell suspension because of their morphology. Some clumps of loosely adhering cells were obtained after trypsinization, and these cells were used for the aggregation (Fig. 1b).

In total, 18 trials were carried out to produce aggregates of 137-cells with tetraploid embryos (Table 1). The fusion rate of 2-cell embryos was 75.5% (723/957); 174 (24.1%) of fused tetraploid embryos developed to the compacted or compacting morula stage, and 28 (36.4%) of 77 aggregates developed to blastocysts. Twelve aggregates that developed to blastocysts were transferred to 7 recipient cows (Table 2). Four of 7 recipient cows became pregnant, and 6 calves representing 2 singletons and 2 sets of twins were produced. The birth weight of the first calf (Exp. No. 4, Calf 5.3 in Table 2) was 57 kg, which is approximately twofold the normal birthweight expected for Japanese black calves; however, this animal was stillborn because of difficulties during delivery. The birth weight of the last calf (Exp. No. 16, Calf 9.4 in Table 2) was 30 kg; however, this female calf died shortly after birth because of excessive bleeding from the umbilicus. The remaining calves are apparently normal and healthy. One set of twins consisted of a phenotypic male and female, and the remaining set of twins consisted of 2 phenotypic males. Chromosome analysis of the male and female twins showed their sex chromosome complement to be a mix of XX and XY, and chromosome numbers were a mixture of diploid and tetraploid cells. Chromosome analyses from the male twins showed diploid cells with XY. All calves showed black hair,

suggesting that the origin is from the tetraploid embryos and not from the 137-cells (Fig. 2). The results of microsatellite analysis of genomic DNA from the chimeric calves using 12 different primer sets are summarized in Figure 3 and Table 3. The DNA spectra using the TGLA122 primer set illustrated in Figure 3 shows that ES-like cells are involved in chimera formation in all tissues presented here. Among the 6 calves, only 2 were chimeric (Calf 5.3 in the liver and Calf 9.4 in the hair roots), although ES-like cells contributed to the placenta in Calves 5.9–1 and 7.2–1. However, some of 12 markers used, in fact up to half (6/12), were not detected in the chimeric tissue (Table 3). Contribution of ES-like cells was not observed in the testes from the 3 male calves.

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TABLE 1. Development of tetraploid embryos aggregated with 137-cells

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FIG. 2. Chimeric calves derived from aggregation of the bovine ES-like cells with tetraploid embryos. These calves were chimeric according to the results of microsatellite analysis

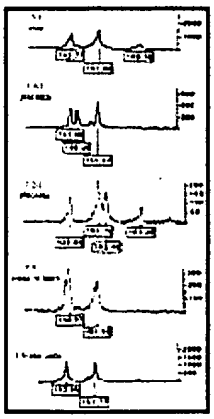

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FIG. 3. DNA spectra of each tissue or ES-like cell by microsatellite analysis using TGLA122 primer. The numbers in boxes and the scale on the Y axis show the lengths and the concentration of DNA fragments amplified by PCR

View this table: [in this window] [in a new window] TABLE 3. Microsatellite DNA analysis of tissues from chimeric calves using 12 primers[a]

DISCUSSION

We have produced chimeric calves from bovine ES-like cells (137-cells) aggregated with tetraploid embryos as judged by microsatellite DNA analysis although the calves were not clones. The results clearly indicate that the 137-cells are able to contribute to chimera formation, but at a very low level. A number of factors may account for these results. First, the proliferation rate of the 137-cells was extremely slow compared to that characteristic of murine ES cells. Bovine 137-cells were derived from ICM cells of Holstein cattle and during these studies were cultured from passage 8 to 15. For aggregation, the 137-cells were cultured on gelatinized dishes to confluence in the absence of feeder layers. The morphology of the 137-cells was unlike murine ES cells: they were flat and epithelial in appearance, similar to other cell populations isolated from sheep and pig embryos [23–26]. The previously reported ovine embryo-derived cell line (TNT4) was cultured in medium supplemented with 0.5% FBS in order to induce to quiescence before nuclear transfer [6]. In nuclear transfer-reconstructed embryos, the oocyte cytoplasm may contain factors able to reprogram quiescent nuclei to control development [6,7]. In this study, however, we did not induce quiescence in the 137-cells by serum deprivation. 137-cells were cultured to confluence on gelatinized dishes in order to obtain some clumps of cells after the trypsinization. Cibelli et al. [18] produced transgenic bovine chimeric offspring using bovine embryo-derived ES-like cells at passage 10 and reported that the ES-like cells contributed to gonadal tissue of the offsprings. In this study, 137-cells did not contribute to testis.

Second, there is a possibility that the electrofused embryos may not be completely tetraploid. In a previous study, 14.3% of morula embryos that developed after electrofusion of embryos at the 2-cell stage were diploid, not tetraploid [19]. These diploid cells in morula embryos may have overcome the other tetraploid cells and the 137-cells. Nagy et al. [12] selected 4-cell-stage embryos after electrofusion for aggregation of murine tetraploid embryos and ES cells; they obtained viable mice that were entirely ES cell-derived. As described in the previous paper [19], when putative tetraploid embryos at the completely compacted morula stage were used for aggregation, it was difficult to form an aggregate. Therefore, in this study, we selected precompaction 8-cell-stage embryos for use in aggregation. However, it was assumed that putative tetraploid embryos after the electrofusion at the 2-cell stage did not develop as perfect tetraploid embryos as in mice. In preliminary analyses of chromosome constitution in the presumptive tetraploids, diploid blastomeres were observed. One explanation of this is that electrofused embryos may not develop as tetraploid because of cytoplasmic and nuclear division

occurring in the absence of DNA replication.

In 4 of the 6 newborn calves, the 137-cells did not contribute to form the body although the placenta showed chimerism. This result suggests that the condition of the clump of 137-cells and the positional relationship with the tetraploid embryos may influence the contribution of the 137-cells to the ICM or trophectoderm. In the previous paper, we investigated the effects of the degree of clumping of donor cells, using ICM cells and mammary cells, and the positional relationship with tetraploid morula embryos on the efficiency of incorporation into the ICM [19]. If the donor cells formed a tight clump, it seems that these cells would contribute at least parts of ICM of the aggregates. It was assumed that the positional relationship of 2 tetraploid embryos and a clump of donor cells in a droplet are very important to produce complete aggregates. Hillman et al. [27] showed that each blastomere of 4-cell-stage mouse embryos placed on the outside surface of a mass of aggregated blastomeres formed a trophoblast. Therefore, they indicated that whether a cell can form an ICM or not was dependent upon the internal or external position after compaction. Nagy et al. [11,12] used an alternative method of aggregation in the mouse, the "Sandwich method," in which ES cells are located between 2 tetraploid embryos for aggregation. They showed that tetraploid embryos contributed to an extra-embryonic membrane [11], and complete ES cell-derived offspring were produced [12]. In cattle, as described previously [19], each putative tetraploid embryo tended to develop individually in the case of the Sandwich method. Therefore, we attempted to put the clump of the 137-cells into a depression to form a triangle with 2 putative tetraploid embryos. Microsatellite analysis indicated that the 137-cells contributed to placenta as well. One of the problems encountered was the loose connection between each of the 137-cells in the clump, which made it difficult to sandwich the clump between of the 2 tetraploid embryos. Consequently the 137-cells as outer cells contributed to the placenta. The birthweight of Calf 5.3, Exp. No. 4, was twice the average birthweight of Japanese Black cattle. However, in murine aggregation chimera, there are no reports showing abnormal birthweight from the aggregation using 2 or 3 embryos [11,12,14,28,29]. Consequently, the cause of increased birthweight of the calf derived from ES-like cells aggregated with 2 embryos is unclear. However, there were 2 abnormal calves: one was stillborn and another one died shortly after birth. Nagy et al. [12] suggested that minor genetic changes or changes in DNA methylation and imprinting in the ES cells as a result of culture underlie phenomena such as a decline in developmental potential. Accordingly, further investigation is needed to clarify the cause of increased birthweight.

The putative tetraploid embryos did not develop as complete tetraploid embryos, and this resulted in the production of chimeric calves. However, cloned animals would be produced by the aggregation technique if it were possible to identify complete tetraploidy before aggregation. In addition, it seems that we need more experiments on aggregation of bovine parthenogenetic embryos, instead of tetraploid embryos, and bovine ES-like cells or quiescent somatic cells, instead of ES-like cells.

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FOOTNOTES

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△ TOP
△ ABSTRACT
△ INTRODUCTION
△ MATERIALS AND METHODS
△ RESULTS
△ DISCUSSION
▫ REFERENCES

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Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells

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We have developed a method, using nuclear transplantation, to produce transgenic embryonic stem (ES)-like cells from fetal bovine fibroblasts. These cells, when reintroduced into preimplantation embryos, differentiated into derivatives from the three embryonic germ layers, ectoderm, mesoderm, and endoderm, in 5-month-old animals. Six out of seven (86%) calves born were found to be chimeric for at least one tissue. These experiments demonstrate that somatic cells can be genetically modified and then dedifferentiated by nuclear transfer into ES-like cells, opening the possibility of using them in differentiation studies and human cell therapy.

Keywords: agricultural biotechnology, nuclear transplantation

Embryonic stem (ES) cells have been available for several strains of mice for many years and have been shown to be capable of contributing to each of the tissues of the animal when combined with a host embryo to form a chimera. Techniques have been developed for inducing the differentiation of mouse ES cells in vitro and successfully transplanting them into recipient mice[1,2]. Success in developing pluripotent cell lines from large animal species, such as bovine, has been minimal. Production of putative bovine ES cells was first reported by Saito et al.[3] and later, a similar type of stem-like cell was reported to direct development through organogenesis[4]. Bovine ES cells that are capable of complete differentiation to term, in vivo, have not been reported. Little success has been achieved in inducing ES cells to differentiate into a specified tissue in vitro or in selecting specific cells, out of the many other types of cells that are present, following the induction of in vitro differentiation.

The objectives of this study were to develop an efficient procedure for producing bovine ES-like cells, to test the pluripotency of these cells in vivo by forming chimeras with host embryos, and to develop an efficient method for genetic modification of the cells using somatic cell nuclear transplantation.

Results

Production of transgenic embryo-derived pluripotent ES-like cell colonies. As one approach to producing transgenic cattle, putative bovine ES-like cells were derived from embryos. In vitro maturation and fertilization of oocytes and in vitro culture of the embryos to the blastocyst stage produced 49 embryos at day 7. Blastocysts were mechanically dissected and plated on mitotically inactivated fetal mouse fibroblast feeder layers. Twenty-seven inner cell masses attached to the feeder layer grew as ES-like cell colonies and successfully survived passaging over at least 12 months without differentiation. These colonies had well-defined edges. Cells in these colonies had a high nuclear to cytoplasmic ratio and a high density of cytoplasmic lipid granules, and were negative for cytokeratin and vimentin. Unlike mouse ES cells, bovine ES cells eventually formed single layer sheets (Fig. 1A) and were alkaline phosphatase negative.

The method of producing transgenic bovine ES-like cells also differed from procedures used for the mouse (Fig. 2A). Bovine ES-like cells, unlike mouse ES cells, do not survive replating when trypsinization is performed; therefore, mechanical passage was used instead. Passage of the cells mechanically involves removing a group of cells, containing a minimum of 50 to 100 cells, and transferring these to fresh feeder layers. Because single cell suspensions could not be passaged, it was not possible to use electroporation for DNA transfection or to clonally propagate transgenic cells. Therefore, microinjection of DNA into the nucleus of individual cells was used as an alternative method. Approximately 500 to 1000 cells could be injected per hour, and injection volume was based on nuclear swelling. Three different cell lines were used. A cytomegalovirus (CMV)-β-galactosidase-neomycin (β-Geo) cassette was delivered into the nucleus of ES-like cells. Five, three, and zero stable, G418 selected transgenic colonies were produced out of 3753, 3508, and 3502 injected cells, respectively. We did not determine if these colonies were derived from single or multiple transgenic cells. During G418 selection the original colony essentially disappeared before growth of the transgenic cells began, indicating a possible clonal origin; however, the possibility of having produced a transgenic colony from two or more closely placed transgenic cells cannot be ruled out. β-galactosidase expression was consistently high in all colonies, although not all cells within a colony expressed the gene (Fig. 1B). PCR amplification of a segment of the transgene also confirmed that the cells were transgenic (Fig. 1E).

Production of transgenic somatic cell-derived bovine pluripotent ES-like cell colonies. Although transgenic ES-like cells can be produced by microinjection, the generation of a large number of transgenic ES-like cells and clonal propagation was not achieved. Therefore, we took another approach (Fig. 2B) that involved transfection of bovine fetal fibroblasts and fusion of the transgenic fibroblast cells to enucleated oocytes to produce blastocyst stage nuclear transplant embryos. These embryos were then plated on fibroblast feeder layers to produce transgenic ES-like cell colonies. Bovine fibroblasts were obtained from a 55-day fetus, and grown and transfected by electroporation using standard methods (Fig. 1C). Three hundred and thirty enucleated mature bovine oocytes were reconstructed with actively dividing fibroblasts. Thirty-seven (11%) blastocysts (day 7.5) were obtained and ES-like cell lines were established from 22 (59%) of these. Out of 22 cell lines, 21 were positive for the transgene after PCR amplification of the β-galactosidase fragment. The negative ES-like colony could have originated from a neomycin-resistant fibroblast that lost the β–galactosidase gene. Fibroblast-derived ES-like cell colonies showed morphology and cytoplasmic marker characteristics identical to those of embryo-derived ES-like cells (Fig. 1D). Furthermore, colonies were passaged for several months without differentiation, even, in one case, when a colony was derived from a senescent, nondividing fibroblast cell line.

Production of chimeric calves. In order to determine the potency of

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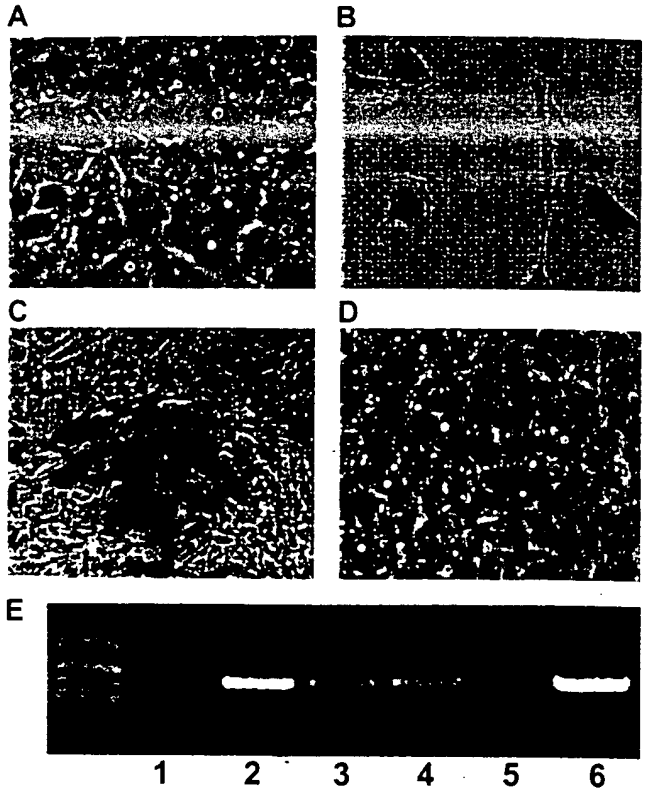

Figure 1. Generation of transgenic ES-like cells. (A) Embryo-derived ES-like cells. (B) β-galactosidase activity of transgenic embryo- derived ES-like cells. (C) β-galactosidase activity of transgenic fetal fibroblasts. (D) Nuclear transfer-derived ES-like cells. (E) PCR ethidium bromide gel of β-galactosidase fragment. Lane 1 : nontransgenic embryo-derived ES-like cells; lane 2: transgenic embryo-derived ES-like cells; lane 3: transgenic fetal fibroblasts; lane 4: transgenic nuclear transfer-derived ES-like cells; lane 5: nontransgenic fetal fibroblasts; lane 6: template.

Table 1. production of transgenic calves using embryo. and NT-derived ES like cells.

	Injected embryos	Blastocyst produced (%)	Blastocyst transferred	Calves born	Transgenic Calves*
Embryo ES-like cells	70	16 (23)	16	5	3
NT ES-like cells	99	22 (22)	10	7	6

*Animals with al least one transgenic tissue

bovine embryo- (passage 10) and fibroblast -derived ES-like cells (passage 3) in vivo, 8 to 10 cells were introduced into day 3 in vitro produced embryos, cultured in vitro until day 7.5 and transferred into synchronized recipients. Five calves were born from embryos that received transgenic embryo-derived ES-like cells, and seven calves were born from embryos that received transgenic nuclear-transfer (NT)-derived ES-like cells (Table 1). All the animals were phenotypically normal.

All the animals were slaughtered at 5 months of age, with the exemption of calf 904, which was killed at 45 days of age. Genomic DNA was isolated from a spectrum of tissues (skin, muscle, brain, liver, spleen, kidney, heart, lung, mammary gland, intestine, and gonads) from each animal, amplified using β-Geo primers, and probed using standard-protocol Southern blot analysis. Results were positive in at least one tissue in nine calves and in two or more tissues in six calves. Oocytes were found to be positive in one animal (Fig. 3). The limited presence of transgenic cells in the newborn animals could be attributed to the fact that not all the ES-like cells were incorporated into the developing morulas; moreover, among those cells that did incorporate, degree of pluripotency may have varied.

Fluorescent in situ hybridization (FISH) analysis was performed in spleen tissue from calf 911 (Fig. 4A), and testis of calf 903 (Fig. 4C). Positive hybridization signals were identified in both tissues. In the spleen, 32% of nuclei (82/256) exhibited green signals compared with negative spleen in which only 1% of nuclei (2/231) were classified as carrying green signals. Testis specimens were not presented as a single monolayer of cells; therefore, percentage of positive cells was not assessed; however, positive signals were detected inside the seminiferous tubules.

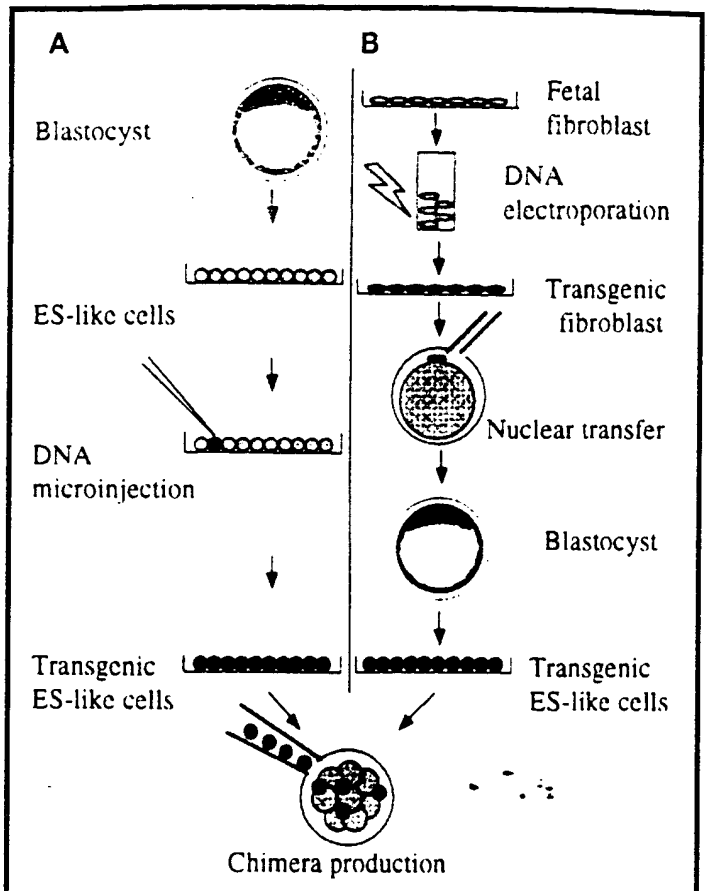

Figure 2. Generation of transgenic ES-like cells (A) by microinjection and (B) by somatic cell nuclear transfer.

Discussion

The first objective of this study was to produce bovine pluripotent ES-like cells. ES-like cells are derived from an early stage embryo or the inner cell mass (ICM) directly, and, therefore, should retain the morphology and cellular characteristics of the ICM. In the mouse, ES cells grow as colonies with a defined margin, and cells have high nuclear to cytoplasmic ratio and high density of lipid inclusions similar to the ICM. Our bovine cells derived both from embryos and NT fibroblasts, also retained these characteristics. The expression of various cytoplasmic markers has also been used to indicate an ICM-like quality of mouse ES cells. In the bovine, ES-like cells derived either from embryonic or somatic cell sources, do not express differentiation markers such as vimentin and cytokeratin in a pattern similar to the ICM; however, these cells are alkaline phosphatase negative. The second characteristic of a pluripotent embryonic cell is that it can be grown over many passages without showing signs of differentiation. In this study, and other preliminary work[5], bovine ICM-derived cells were passaged for over 1 year without losing the morphological and cellular similarities to the ICM. The third and most important characteristic used to define ES-like cells is that, upon introduction into a preimplantation embryo, they are able to colonize the ectodermal, mesodermal, and endodermal tissues and the germ line, as the host embryo develops and differentiates. In this study it was shown that both embryonic and fibroblast-derived ES-like bovine cells are capable of giving rise to multiple tissues in 5-month-old animals. Our results demonstrate that cells derived from somatic and embryonic sources possess functional and phenotypic characteristics of pluripotent ES-like cells.

Much work has been done in many different species toward developing methods of producing ES cells; however, little success has been reported at meeting all the criteria listed above. In rabbit[6], production of chimeric offspring was reported, but no chimerism in gonads was demonstrated. In hamster[7,8] and cow[3,4], cells were grown in vitro; however, no chimeric animals were

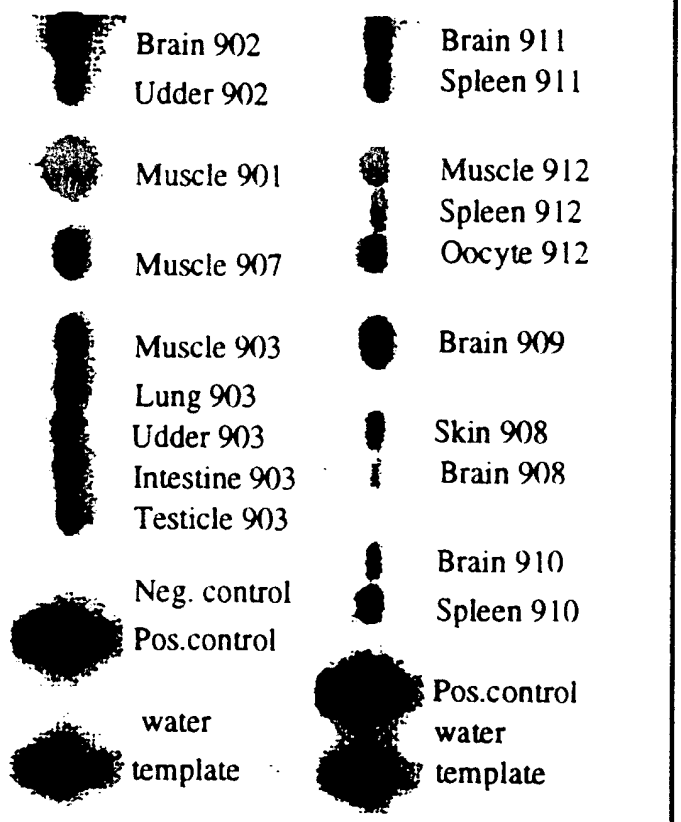

Figure 3. Southern blot analysis of PCR-amplified products of tissues from chimeric calves. Calves 901 to 903 were generated from embryo-derived ES-like transgenic cells. Calves 907 to 912 were generated from nuclear transfer-derived ES-like transgenic cells.

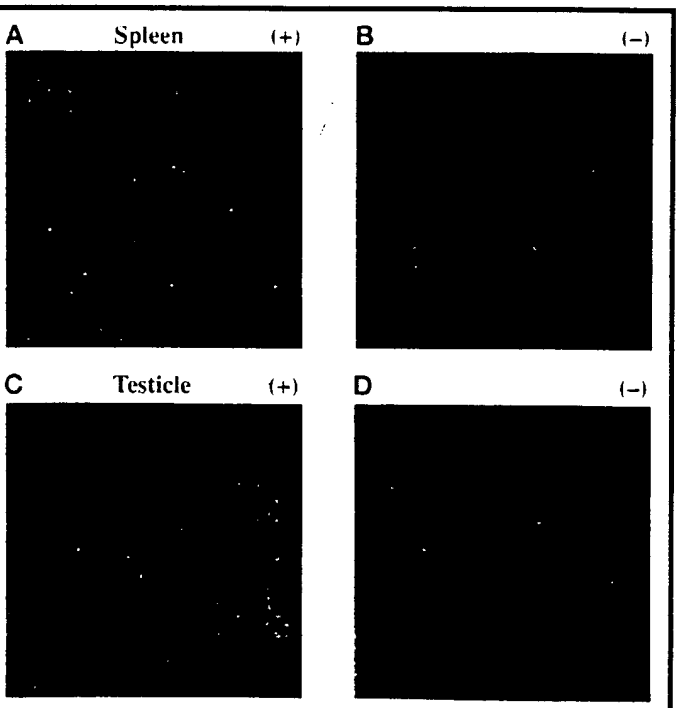

Figure 4. FISH analysis of (A) spleen from calf 911 produced with NT-derived ES-like cells, (B) spleen, negative control, (c) testis of calf 903 produced with embryo-derived ES-like cells, and (D) testis, negative control.

produced. This is the first published report demonstrating transgenic chimerism in full-term live mammals, including in gonadal tissue from a species other than the mouse. However, until germline transmission is demonstrated, we refer to our cells as "pluripotent or ES-like cells" instead of ES cells. The results in this study indicate that, although genetic modifications could be made directly in bovine ES-like cells by microinjection, and transgenic cells could be selected by a standard neomycin resistance approach, limitations in the number of cells that can be microinjected, the slow growth of the cells, and our inability to clonally propagate the ES-like cells limits the usefulness of this approach, particularly for gene targeting. This is one important difference between bovine ES-like cells and mouse ES cells. Aside from the fact that care must be taken to prevent differentiation, mouse ES cells can be readily grown in culture, clonally propagated, transfected by standard high-volume gene transfer methods, and in many cases, exhibit high-efficiency homologous recombination. In our system, the low transfection efficiency with of bovine ES-like cells prevents the possibility of using direct ES-like cell transfection for gene targeting.

An alternative method of making genetic modifications in bovine ES-like cells is to genetically modify fibroblast cells and then produce embryos by nuclear transplantation. Genetic modification is relatively simple with fibroblast cells, which are easy to grow, transfect, and clonally propagate. Furthermore, gene targeting and selection for homozygous lines in vitro have been successful in human fibroblast lines[9].

This study demonstrates that ES-like cells can be produced from bovine embryos, which can be cultured without a change in morphology for indefinite periods in vitro and retain the ability to give rise to tissues derived from all three germ layers in offspring. Furthermore, using nuclear transplantation, these cells can be produced from genetically modified fibroblasts. This system could be useful for the in vitro production of genetically modified bovine cells to be used for cell transplant therapies for many different human diseases.

Experimental protocol

In vitro maturation of bovine oocytes. Ovaries were recovered at a slaughterhouse, placed in warm phosphate-buffered saline (PBS) (34°C) and brought to the laboratory within a limit of 8 h. Each follicle of more than 2 mm in diameter was aseptically aspirated with an 18 gauge needle. Search of oocytes was performed in modified Tyrode's medium (TL Hepes). Oocytes with a homogeneous cytoplasm, considerable perivitelline space and intact cumulus cells were placed in maturation medium M199 (GIBCO, Grand Island, NY), 10% fetal calf serum (FCS), 5µl/ml bovine follicle-stimulating hormone (Nobl, Sioux Center, IA), 5µl/ml bovine luteinizing hormone (Nobl), and 10µl/ml Penstrep (Sigma, St. Louis, MO) for 22 h at 38.5°C and 5% CO_2.

In vitro fertilization of bovine oocytes. Twenty-two hours postmaturation, oocytes were placed in fertilization medium (5 ml CR2-Specialty medium, stock solution 100 U/ml penicillin, 100µg/ml streptomycin, 0.005µg/ml phenol red, 30 mg bovine serum albumin fatty acid free, 5µg/ml sodium heparin). A unit of frozen semen was thawed and placed on top of a percoll layer that contains 90% percoll (Sigma) and one part 10X modified sperm TL plus, 45% percoll (one part of 90% percoll stock solution and one part sperm TL without BSA). Dead sperm were separated from live sperm by centrifugation at 700 G for 30 min. Sperm pellet was resuspended at a final concentration of 500,000 sperm/ml. After 12 h in culture at 38.5°C and 5% CO_2, eggs were removed and placed in CR2 medium with 3 mg/ml BSA.

Embryo culture. During the first 3 days after fertilization, embryos were cultured in 500µl well plates with mouse embryonic fibroblast (MF) feeder layers and CR2 with 6 mg/ml BSA. On day 4, embryos were transferred to 500µl well plates with MF feeder layers, CR2 with 6 mg/ml BSA, and 10% FCS until blastocyst stage (day 7 postinsemination).

ES-like cell culture. Blastocysts were placed in a 32 mm plate (Nunc, Rochester, NY) with mitotically inactivated MF feeder layer and ES medium (Alpha MEM, 10% fetal calf serum, 4µl/ml antibiotic-antimycotic, 2.8µl/ml 2-mercaptoethanol, 0.3 mg/ml L-glutamine, and 1µl/ml tylosin tartrate) equilibrated a day in advance at 38.5°C and 5% CO_2. Using a 22 gauge needle, blastocysts' zona pellucida and trophoblast were mechanically removed. The remaining ICM was placed underneath the MF. After 1 week in culture, ES-like cells were passaged to a fresh mitotically inactivated MF. Inactivation of MF was performed by exposing them to gamma radiation (2956 rads). ES-like cells were passaged by cutting a small piece (50 to 100 cells) of the colony and placed on top MF feeder layers using a pulled Pasteur pipette.

Nuclear transplantation. Eighteen hours postmaturation, oocytes were placed in a 100µl drop of TL HECM-Hepes under mineral oil (Sigma). Oocyte enucleation (extraction of chromosomes) was performed using a beveled glass pipette of 25µm diameter. Evaluation of enucleation was done by exposure of individual oocytes previously cultured for 15 min in 1µg/ml of bisBENZIMIDE (Hoechst 33342; Sigma) in TL HECM-Hepes under ultra-violet light. Donor cells were placed in the perivitelline space and fused with the egg's cytoplasm at 23 h postmaturation. Oocytes and donor cell were placed into 4 ml medium made of 50% SOR2 fusion medium (0.25 M D-sorbitol (Sigma), 100 µM CaOAc (Sigma), 0.5 mM magnesium acetate (Sigma), 1.0 g BSA (Sigma), and 50% HECM-Hepes for 2 min. Eggs were then placed between the electrodes of a 500µm fusion chamber. Once the eggs were aligned, a pulse of 90 V was administered over 15µs. Eggs were then returned to the 50/50 medium of SOR2 and HECM/Hepes for 2 min and, finally, placed

into a 500µl drop of CR2 at 38.5°C and 5% CO_2 until activation.

Oocyte activation. Activation was performed in general as described[12]. Briefly, 25 to 27 h postmaturation oocytes were incubated in 5µm ionomycin (Cal Biochem, La Jolla, CA), and 2 mM of 6-dimethylaminopurine (DMAP; Sigma) in CR2 with 3 mg/ml of BSA (fatty acid free; Sigma). After activation, eggs were washed in HECM/Hepes five times and placed for culture in a 500µl well of MF and CR2 with 3 mg/ml of BSA (fatty acid free) at 38.5°C and 5% CO_2.

Transgenic ES-like cell production. Five micrograms per milliliter of a β-Geo cassette gene were microinjected into the nuclei of bovine ES-like cells. Twenty four to forty-eight hours after microinjection, 150µg/ml of G418 was added to the culture medium. After 3 weeks under selection, a colony was considered transgenic upon DNA screening by PCR and ethidium bromide gel, and by β-galactosidase staining.

Bovine fibroblast production and electroporation. Bovine fibroblasts were produced from a 55-day-old fetus as follows. Under sterile conditions, the livers, intestines, and heads of the fetuses were discarded. The remaining parts of the fetuses were carefully minced and placed in a solution of Delbucco's phosphate buffered saline (DPBS) with 0.08% trypsin (Difco, Detroit, MI) and 0.02% EDTA (Sigma). After 30 min incubation at 37°C the supernatant was discarded and the pellet resuspended with trypsin- EDTA/DPBS. After 30 min incubation, the supernatant was removed and centrifuged at 300 G for 10 min. The pellet of cells was then resuspended with ES culture medium and plated in polystyrene tissue culture dishes (25010; Corning, Charlotte, NC). After two passages, cells were electroporated with a β-Geo cassette gene with the protocol described by Invitrogen (San Diego, CA) for COS cells[11]. After 3 weeks under 400µg per ml of G418 selection, fibroblasts were considered transgenic upon DNA screening by PCR and ethidium bromide gel, and by β-galactosidase staining.

Alkaline phosphatase staining. Culture medium was removed from the plates and cells were fixed with 4% paraformaldehyde for 20 min. Cells were washed three times in Tris-maleate buffer (3.6 g Trizma base [Sigma], in 1 L water, pH raised to 9.0 with 1 M maleic acid) for 10 min each wash. The last wash was removed and the staining solution (Tris-maleate buffer, 200µl of a 0.5 mM $MgCl_2$, naphthol AS-MX phosphate [Sigma], 0.4 mg/ml, Fast blue [Sigma], 1 mg/ml) was added to the cells for 15 to 20 min. Once blue cells were detected, the reaction was stopped by adding PBS which brought the pH to 7.4.

Chimera production. Seventy-two hours after in vitro fertilization (eight cell stage), embryos were placed in manipulation medium (HECM/Hepes with 10% FCS and 7.5µg /ml of cytochalasin B [Sigma]). ES-like cells were dissociated using 0.08 % trypsin (Difco) and 0.02% EDTA in PBS during 25 to 30 min. Using a 15-20µm diameter beveled pipette, 8 to 10 cells were introduced into the embryos. Embryos were placed in a 500µl culture drop (MF feeder layer, CR2 with 6 mg/ml of BSA and 10% FCS).

Immunohistochemical studies. Primary antibodies specific against cytokeratin 8-18 (Sigma) and vimentin (Sigma) were used in ES-like cell cultures. Cells were plated on sterile glass slides, fixed in 2% paraformaldehyde, and extracted with cold (-20°C) acetone. Cells were incubated with primary anti-body dilutions in PBS containing 0.5% BSA (PBSA) for 1 h at room temperature. Slides were then rinsed three times in PBSA with changes of rinse solution every 10 min, and incubated for 1 h in fluorescent 5-isothiocyanate (FITC) conjugated antimouse IgG (Sigma). After rinsing in PBSA for 30 min, coverslips were mounted in 50% glycerol and observed under a fluorescence microscope[8].

β-galactosidase staining. Culture medium was removed from the plates, and cells were fixed with 2% glutaraldehyde in PBS. Then cells were washed three times with PBS and color substrate (5 mM $K_3Fe(CN)6$, 5 mM $K_4Fe(CN)6$, 1 mM $MgCl_2$, 1 mg/ml X-gal in PBS, pH 7.0-7.5) was added for 3 h[12].

PCR analysis and blot analysis. Analysis of transfected cells and tissue from 5-month-old animals was performed using a sense primer (ACT3βGeo, a 21 base CGCTGTGGTACACGCTGTGCG) and antisense primer (ACT4βGEO, a 22 base CACCATCCAGTGCAGGAGCTCG [Amitof Biotech, Boston, MA). Reactions were run for 35 cycles (1) heated at 95°C for 30 s (2) primers were annealed at 65°C for 1 min, (3) extended for 2 min at 72°C, followed by 10 min extension at 72°C. The amplified product was a 782 bp fragment. Sample analysis was performed by separating by size in a (1%) TAE agarose gel electrophoresis containing ethidium bromide. Products were sized by comparison with markers consisting of 1444 bp, 943 bp, 754 bp, 585 bp, 458 bp, 341 bp, 258 bp, 153 bp, and 105 bp. DNA was then handled according to standard Southern blot analysis protocols. Briefly, DNA was transferred to Zetabind (Cuno, Meriden, CT) by capillary transfer and probed with a gel-purified 289 bp ClaI to EcoRV fragment labeled with ^{32}PdCTP using random primed labeling kit (Boehringer Mannheim, Indianapolis, IN). Hybridization was done at 42°C overnight. After washing, the blot was exposed to Biomax film (Kodak, Rochester, NY) overnight. Nontransgenic fibroblasts and water were used as negative controls, and transgenic cells for β-Geo and template were used as positive control. When oocytes were analyzed, ovarian follicles were aspirated with a syringe using an 18 gauge needle. Eggs' granulosa cells were removed by vortexing the oocyte/cumulus cell complex in 5 mg/ml of hyaluronidase (Sigma) in PBS. Oocytes were washed five times in PBS before DNA isolation.

FISH analysis. Samples were frozen and made onto slides either by slightly pressing the sample against the slide (for spleen slides) or by cryosections (for testis slides). β-Geo DNA was linearized with ScaI and biotin-labeled by nick translation reaction. An aliquot of the biotin-labeled DNA was run on a gel and transferred to a membrane, and a streptavidin-alkaline phosphatase assay was performed to detect the size of labeled fragments and quantity of biotin incorporation. The labeled DNA was then coprecipitated with salmon sperm DNA as carrier. A number of single-target single-color FISH assays were performed using varied concentrations of labeled DNA as a probe (250-500 ng). The specimens were washed in 70% acetic acid and digested in pepsin (0.01% in 0.01M HCl at 37°C) before denaturation. Testis slides were incubated in pepsin at room temperature for 10 min before warming to 37°C. Denaturation was performed at 75°C for both chromosomal and probe DNAs and hybridization was allowed to occur for approximately 60 h. Posthybridization washes included three 5 min washes in 50% formamide/2XSSC and three 5 min washes in 2XSSC at 43°C. Immunochemical detection was achieved with consecutive incubations in FITC-avidin, biotinylated anti-avidin and FITC avidin (Vector, Burlingame, VT). Chromatin was counterstained with DAPI (0.01µg /ml on antifade; Boehringer Mannheim). After hybridization, slides were coded and blindly analyzed. Analysis was performed in an Olympus BX-60 fluorescence microscope using interference filter sets for single band (DAPI and FITC) and triple band (DAPI, FITC, Texas red). Gray images were acquired using a CCD camera (Photometrics, Phoenix, AZ) and combined using the Oncor (Gaithersburg, MD) image software.

Acknowledgments

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Modification of the mouse mitochondrial genome by insertion of an exogenous gene

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Abstract

Using homologous recombination in yeast we have inserted a synthetic gene encoding human ornithine transcarbamylase (s*OTC*), designed to allow mitochondrial (mt) translation, into the mouse mt genome. Modification of the mt genome was facilitated by its cloning into a yeast centromeric plasmid. The s*OTC* gene was initially flanked by 25 bp of the mt $tRNA^{His}$ gene at its 5′ end and by 23 bp of the mt $tRNA^{Ser\ (AGY)}$ gene at its 3′ end (Wheeler et al., 1996). In order to achieve homologous recombination the flanking homology was subsequently extended to 525 and 362 bp by the polymerase chain reaction (PCR). The s*OTC* gene was thus inserted into the cloned mt genome at a unique location between the $tRNA^{His}$ and $tRNA^{Ser\ (AGY)}$ genes. Positioning of the s*OTC* gene between these normally contiguous tRNA genes should allow its processing from the mt polycistronic transcript. The ability to modify the mammalian mt genome in this way is a valuable step towards a functional analysis of mt genetic mechanisms and possibly also towards a gene therapy approach for mt disorders. © 1997 Elsevier Science B.V.

Keywords: Synthetic gene; Ornithine transcarbamylase; Homologous recombination; *Saccharomyces cerevisiae*; Mitochondrial disease

1. Introduction

The mammalian mitochondrial (mt) genome is a 16–17 kilobase (kb) double-stranded circular DNA molecule. The genome is very economically organised, being essentially saturated with coding sequences and containing only one major 1 kb non-coding regulatory region, the displacement loop (D-loop) (Anderson et al., 1981; Bibb et al., 1981). It contains 13 protein-coding genes, specifying respiratory chain subunits, as well as the 22 tRNA genes and two rRNA genes required for their translation by the mt translation system. Defects in the mt genome play an important and increasingly apparent role in human disease (Shoffner and Wallace, 1990). An understanding of both the general mechanisms of replication and expression of this genome and the molecular pathology of mtDNA disorders would be facilitated by the ability to analyse the effect of changes specifically introduced into the mt genome.

Modification of the mt genome of the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) can be achieved by biolostic bombardment of whole cells with DNA-coated tungsten particles (Fox et al., 1988; Johnston et al., 1988). This has proven unsuccessful in mammalian systems, possibly owing to the high copy number of endogenous mtDNA, as well as to the absence of extensive recombination mechanisms in mammalian mitochondria, in comparison with the yeast organnelle (Clayton, 1982). An alternative approach may be to modify the mtDNA in vitro, to introduce this DNA into isolated mitochondria (Collombet et al., 1997) and to use these modified mitochondria to repopulate recipient cells (King and Attardi, 1988).

As a test system for such an approach we are investigating the possibility of expressing the normally nuclear-localised gene encoding human ornithine transcarbamylase (OTC; EC 2.1.3.3) from the mouse mt genome. We

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Abbreviations: bp, base pair(s); H-strand, heavy-strand; kb, kilobase(s) or 1000 bp; L-strand, light-strand; mt, mitochondrial; nt, nucleotide(s); OTC, ornithine transcarbamylase; PCR, polymerase chain reaction; s*OTC* gene, synthetic *OTC* gene sequence.

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2.7. Rescue of plasmid DNA

Two agarose plugs of total yeast DNA (about 180 μl), were melted at 65°C with 2 volumes of TE pH 8.0. The DNA was extracted once with phenol, once with chloroform/isoamyl alcohol (24:1). It was then ethanol precipitated at −70°C for 30 min, pelleted by centrifugation, rinsed in 70% ethanol, air-dried and dissolved in 10 μl STE (100 mM NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA). The DNA was used to transform MAX Efficiency DH5α competent *E. coli* cells (Gibco BRL) according to the manufacturer's instructions.

2.8. DNA sequencing

Sequencing of DNA was carried out by the dideoxy chain-termination method (Sanger et al., 1977) using the Sequenase version 2.0 DNA sequencing kit (US Biochemicals, Cleveland, OH, USA), incorporating [α-^{33}P]dATP (Amersham).

3. Results

3.1. Generation of mtDNA homology flanking the sOTC gene

To insert the s*OTC* gene into the mouse mt genome we elected to exploit the efficient mechanisms of homologous recombination in *S. cerevisiae*. The s*OTC* gene, flanked by 25 base pairs (bp) of mouse mitochondrial tRNA^{His} sequence at its 5′ end and by 23 bp of tRNA$^{Ser(AGY)}$ sequence at its 3′ end, was cloned as an *Eco*RI–*Hin*dIII fragment (pGEMOTC) (Wheeler et al., 1996). The aim was to direct the s*OTC* gene into the mouse mt genome between these two normally contiguous tRNA genes, since tRNAs are thought to function as recognition sites for mtRNA processing (Ojala et al., 1981). The length of homology to a target locus is an important factor influencing the efficiency of recombination and has been achieved with sequences as short as 15 bp (Baudin et al., 1993; Manivakasam et al., 1995). However, since it was not possible to select directly for insertion of the s*OTC* gene we decided to extend the region of homology with the mt genome in order to increase the efficiency of homologous recombination.

The mtDNA sequence flanking the s*OTC* gene was extended in three steps by PCR cloning using the mt genome as a template (Fig. 1). In step A the 5′ flanking region was extended from a primer containing the mt tRNA^{His} sequence and a tail of s*OTC* sequence to give clone pmt5′OTC in which the s*OTC* gene was flanked at its 5′ end by 854 bp of mouse mtDNA and at its 3′ end by 23 bp of the mt tRNA$^{Ser(AGY)}$ gene. In step B the 3′ flanking region was extended in a similar way from the mt tRNA$^{Ser(AGY)}$ sequence to give clone pmt3′OTC in which the s*OTC* gene was flanked at its 5′ end by 25 bp of mt tRNA^{His} sequence and at its 3′ end by 362 bp of mtDNA. In step C sequences from these two clones were combined to give pmtEXT, in which the s*OTC* gene was flanked 5′ by 854 bp of mtDNA and 3′ by 362 bp of mtDNA.

3.2. Insertion of the sOTC gene into the mouse mt genome

To insert the s*OTC* gene into the mouse mt genome we first cloned the entire mouse mt genome at its unique *Sac*I site into the yeast centromeric plasmid pRS316 (Sikorski and Hieter, 1989). The integrity of the resulting clone, pRSmt22, was confirmed by restriction analysis and by limited sequencing of the D-loop, origin of L-strand replication and the tRNA^{His} and tRNA$^{Ser(AGY)}$ genes (not shown).

Plasmid pmtEXT was digested with *Hin*dIII to excise a 1.86 kb fragment containing 525 bp (5′) and 362 bp (3′) of mtDNA sequence flanking the s*OTC* gene. This fragment was co-transformed into the AB1380 strain of *S. cerevisiae* with pRSmt22. Transformants were selected on media lacking uracil and uracil-prototrophic (Ura$^+$) colonies screened for the presence of s*OTC* sequence by hybridisation with the s*OTC* gene. 13/195 Ura$^+$ clones (C1–C13) were found to be positive for the s*OTC* gene. Transformation of the s*OTC* fragment alone did not produce any Ura$^+$ clones. Fig. 2 shows the predicted recombination event between the s*OTC* *Hin*dIII fragment and pRSmt22 that would generate the desired construct pRSmtOTC.

To determine whether the s*OTC* gene had recombined at the desired position in pRSmt22 a PCR assay was carried out on clones C1–C13. This employed two primers, one of which (P4560) annealed to the mtDNA outside the recombining *Hin*dIII fragment whilst the other (P4563) annealed to s*OTC* sequence within the *Hin*dIII fragment. These primers amplified a 1460 bp product from clones C1, C2, C3, C4, C6, C7, C8, C9, C12, C13 as would be predicted if homologous recombination had occurred correctly (Fig. 3). Clones C5, C10 and C11 failed to give a product. No product was amplified using pRSmt22 as a template.

A number of clones were further analysed by Southern blot. DNA from clones C1 to C4 was digested with either *Sac*I, *Hin*dIII and *Bgl*II, and probed successively with mtDNA and the s*OTC* gene. The autoradiograph of clone C1 (Fig. 4) shows the expected hybridisation patterns using both mtDNA and s*OTC* probes. Under the conditions used no hybridisation of mtDNA to the 4917 bp *Hin*dIII fragment, which contains only 89 bp of mtDNA, is detected. An identical hybridisation pattern was observed for clones C2, C3 and C4. Southern blot analysis of the remaining clones showed either no

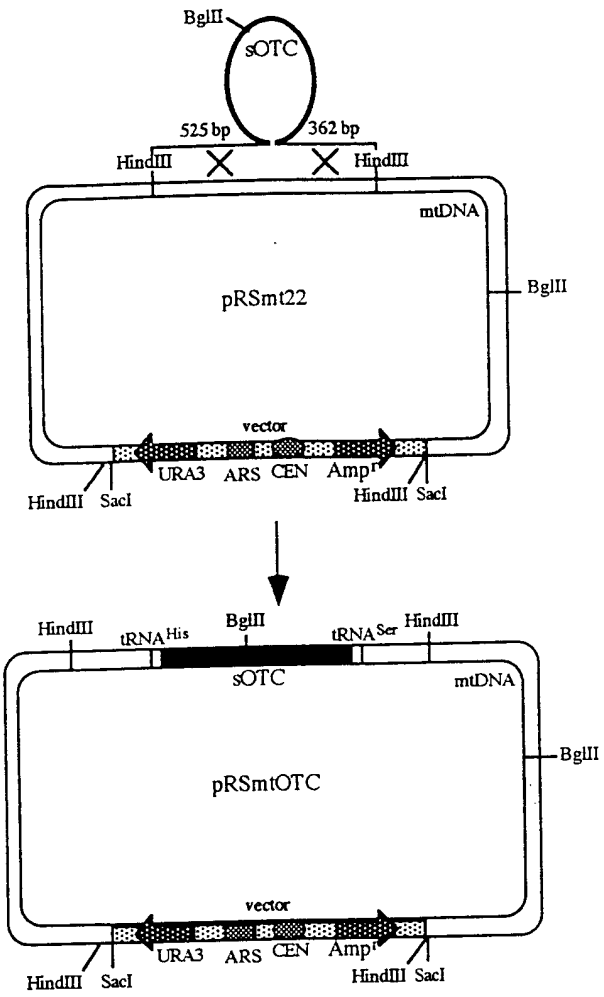

Fig. 2. Insertion of the s*OTC* gene into the mouse mt genome by homologous recombination. Mouse mtDNA (white) is cloned at its unique *Sac*I site into vector pRS316 (grey) to give pRSmt22. Homologous recombination with a *Hin*dIII fragment containing the s*OTC* gene (black) flanked by mtDNA results in the insertion of the s*OTC* gene into pRSmt22 to give pRSmtOTC.

hybridisation signal, conflicting with the PCR results or indicated tandem insertions.

3.3. Rescue of the recombinant plasmid pRSmtOTC

The recombinant plasmid pRSmtOTC from clones C1 to C4 was rescued by transforming *E. coli* with total yeast DNA. Digestion of the purified plasmid with six enzymes (*Sac*I, *Hin*dIII, *Bgl*II, *Pst*I, *Pvu*II, *Eco*RV) produced fragments of the expected sizes.

Since the cloning steps used to extend the mtDNA sequence flanking the s*OTC* gene employed PCR to amplify the appropriate regions of mtDNA, we sequenced the pRSmtOTC recombinants over any PCR-amplified region between the two *Hin*dIII sites used to excise the transforming s*OTC*-containing fragment. Sequencing of clone C2 revealed a single C to T transition (L-strand sequence), at position 11 723 of the

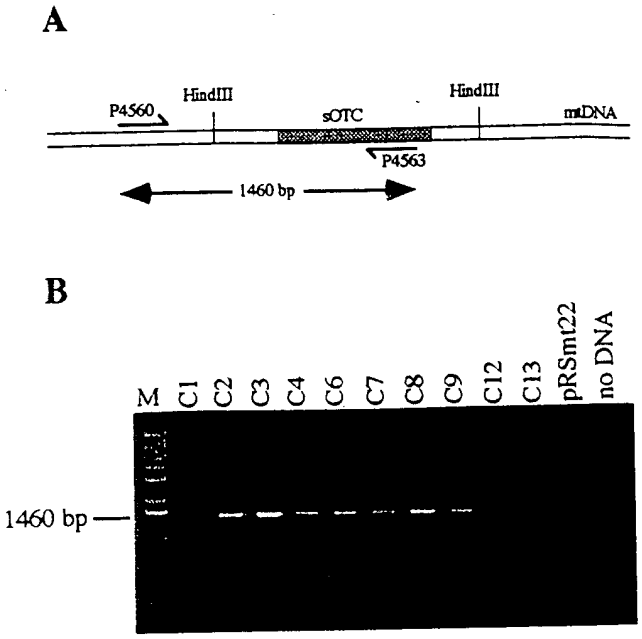

Fig. 3. PCR analysis of recombinant yeast clones. (**A**) Annealing positions of primers P4560 and P4563 and the size of the product amplified with the s*OTC* gene correctly inserted into the mt genome. The *Hin*dIII sites shown are those that flanked the s*OTC* fragment used for the homologous recombination with pRSmt22. (**B**) PCR was carried out with either total DNA prepared from the yeast clones, or with purified plasmid pRSmt22 or no DNA as controls. One-tenth of the PCR product was electrophoresed in an ethidium bromide-stained 1% agarose gel, and visualised by UV irradiation. The molecular weight marker (M) is a 1 kb ladder.

mouse mtDNA, occurring within the mt $tRNA^{Leu(CUN)}$ gene. This mutation was found to be absent in pRSmt22 but present in pmtEXT, suggesting its introduction during the 60 cycle PCR extension of the 3′ flanking mtDNA. Clones C1, C3 and C4 were found to possess a C to T transition (L-strand sequence), also introduced during PCR, at position 11 863 of the mouse mt genome, within the NADH dehydrogenase subunit 5 gene. Clone C2 was fully sequenced over the PCR-amplified regions and no further mutations were found. Importantly, the s*OTC* gene was found to be correctly inserted into the mouse mt genome. Its 5′ end directly followed the mitochondrial $tRNA^{His}$ gene, ending at position 11 606, and its 3′ end directly preceded the mitochondrial $tRNA^{Ser(AGY)}$ gene, beginning at position 11 607.

4. Discussion

We have constructed a plasmid, pRSmtOTC, in which a synthetic gene encoding human OTC (s*OTC* gene) is inserted into the cloned mouse mt genome. This provides the first example of an attempt at specifically engineering this mt genome to contain an exogenous gene. As is the case for a number of other applications (Rothstein, 1991), manipulations of the mt genome that involve the

be shuttled between *E. coli* and yeast, allowing cloning by homologous recombination and subsequent large-scale DNA isolation, the subsequent need to remove vector sequence is a disadvantage of this procedure.

We have thus inserted an additional gene into the mouse mt genome so that it has the potential to be appropriately expressed in mitochondria. We have taken into account both the codon usage of mammalian mitochondria by designing a synthetic *OTC* gene that allows mitochondrial translation, and the mechanism of transcript processing by inserting the s*OTC* gene between two tRNAs. We aim to investigate expression of the s*OTC* gene 'in organello' following mitochondrial electroporation (Collombet et al., 1997). This is a convenient system in which to assay expression that does not rely on the added step of reintroducing the mitochondria into cells. Ultimately, however, the potential for correction of OTC deficiency will be assessed following the transfer, e.g. by microinjection (King and Attardi, 1988), of the modified mitochondria to hepatocytes or oocytes from OTC deficient mice (Doolittle et al., 1974, DeMars et al., 1976). The ability to modify the mt genome has important implications for a comprehensive analysis of mt genetic mechanisms and may provide an interesting new approach towards gene therapy for mt disorders.

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Maternally Transmitted Histocompatibility Antigen of Mice: A Hydrophobic Peptide of a Mitochondrially Encoded Protein

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Summary

MTF, a murine minor histocompatibility antigen, is maternally inherited and thought to be encoded by a mitochondrial gene. We sequenced the entire mitochondrial genomes from three strains that differ in MTF (*Mtf*$^{\beta}$, *Mtf*$^{\gamma}$, and *Mtf*$^{\delta}$) and compared the sequences with the known, *Mtf*$^{\alpha}$, mitochondrial DNA sequence. We found only one site where all four genomes differed, affecting amino acid residue 6 of ND1, a subunit of NADH dehydrogenase. Incubation of non-*Mtf*$^{\alpha}$ target cells with synthetic peptide ND1α1-17 (the first 17 amino acids of the ND1 protein of *Mtf*$^{\alpha}$ mice) rendered them susceptible to lysis by MTF$^{\alpha}$-specific cytotoxic T cells (CTLs). Similarly, non-*Mtf*$^{\beta}$ target cells were lysed by MTF$^{\beta}$-specific CTLs after incubation with the allelic form ND1β1-17. Thus, *Mtf* is attributable to allelic variation at a single residue of the ND1 protein. Cells can therefore display peptides derived from mitochondrially encoded proteins, and such peptides can be histocompatibility antigens.

Introduction

Molecules of the major histocompatibility complex (MHC), such as H-2K and H-2D of the mouse, can be likened to vises that position peptide antigens for presentation to T cells (Bjorkman et al., 1987a, 1987b; Townsend and McMichael, 1987). Recognition of an antigen is restricted by the presenting MHC molecule. MHC molecules themselves are potent (major) transplantation antigens. There are many, probably several hundred, less immunogenic minor histocompatibility (H) antigens. Their encoding genes have been mapped, imprecisely at best, throughout the genome; but their true identity and structure remain a riddle (Loveland and Simpson, 1986; Simonsen, 1987), with the sole exception of β_2-microglobulin in the *H-3* complex (Rammensee et al., 1986; Roopenian and Davis, 1989).

Mta is a minor H antigen made up of the maternally transmitted factor, MTF, whose inheritance implies mitochondrial origin (Fischer Lindahl, 1985), Hmt, and β_2-microglobulin (Fischer Lindahl et al., 1983, 1986, 1987). Cytotoxic T lymphocytes (CTLs) distinguish four allelic forms of MTF: α, β, γ, and δ (Hirama and Fischer Lindahl, 1985). Most inbred strains and wild mice carry *Mtf*$^{\alpha}$; *Mtf*$^{\beta}$ is found in a few strains, while *Mtf*$^{\gamma}$ and *Mtf*$^{\delta}$ appear in some wild mice (Fischer Lindahl, 1986). Unlike other minor H antigens, MTF is recognized by effector T cells that are not H-2 restricted. Rather, the CTLs require the presence of Hmt, the class I–like product of an MHC gene that is distal to *Tla* on mouse chromosome 17 (Fischer Lindahl et al., 1983, 1986; Richards et al., 1989).

The maternal transmission of *Mtf* is stable for at least 20 generations in mice, and it is not affected by foster nursing (Fischer Lindahl and Hausmann, 1983), by embryo transfer to surrogate mothers, or by a long period of cell contact in bone marrow chimeras, thus rendering viral transmission unlikely (Fischer Lindahl and Bürki, 1982). Mitochondrial DNA (mtDNA) is the only stable, maternally inherited genetic element in the mouse, and *Mtf* was found to be transmitted together with the mitochondria (Ferris et al., 1983a). In vitro studies confirmed this linkage: somatic cell hybrids in which mitochondria from one parent were poisoned by rhodamine 6G expressed the MTF phenotype of the untreated parent only (Smith et al., 1983; Huston et al., 1985).

The discovery of *Mtf* raised the problem of how an antigen determined by a mitochondrial gene reached the cell surface. Mitochondria import many proteins encoded in the nucleus, but none of the 13 known proteins encoded by mitochondrial genes or the mitochondrial RNA transcripts is known to leave the mitochondria (Tzagoloff and Myers, 1986). Hence there was no obvious candidate for *Mtf*. But as mitochondrial genomes are highly conserved and restriction enzyme profiles vary with MTF phenotypes (Ferris et al., 1983a, 1983b), we expected that the limited variation in mtDNA sequences could be exploited as the means to pinpointing *Mtf*.

Short synthetic peptides can substitute for native viral and other protein antigens and be readily recognized by antigen-specific helper and killer T lymphocytes (see Möller, 1988). It seemed likely, therefore, that the *Mtf* alleles arose from amino acid changes in a mitochondrial protein sequence long enough to encompass an epitope for T cells, i.e., perhaps 8 to 15 amino acids. Substitutions at a single residue or within a short stretch would suffice and should be evident on comparing mtDNA sequences.

Results

ND1 Protein Sequence Suggests a Candidate for MTF

We sequenced three complete mitochondrial genomes (the β, γ, and δ types) and parts of two others (α[ddY] and α[spretus]) (Yonekawa et al., unpublished data; Wang et al., unpublished data), and compared them with type α (Bibb et al., 1981). The α, β, and γ sequences are quite similar, but δ differs considerably. Most nucleotide changes are silent, and where amino acid substitutions do occur, they are conservative (Table 1).

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Table 1. Amino Acid Substitutions in Mitochondrial Genes

Gene	Length (amino acids)	Base[a]	α	β	γ	δ
ND1	315	**2766-7**	**Ile**[b]	**Ala**	**Val**	**Thr**
		2934	Arg	Cys	Arg	Arg
		3220	Asn	Asn	Asn	Ser
ND2	345	3932	[c] Ala	Thr	Ala	Ala
		3965	Val	Val	Val	Met
		4187-8	Gly	Gly	Asp	Asn
		4371	Ile	Ile	Ile	Thr
		4550-1	Ala	Ala	Ala	Ile
		4581	Asn	Asn	Asn	Ser
		4706	Ile	Val	Val	Ile
		4794	Ile	Thr	Thr	Thr
		4889	Phe	Phe	Leu	Phe
		4947	Thr	Thr	Thr	Ile
COI	514	5463	Ala	Thr	Ala	Ala
		6651	Asp	Asp	Tyr	Asp
		6852	Thr	Thr	Ala	Thr
COII	227	no substitutions				
ATPase 8	67	7847	Val	Val	Ile	Val
		7898	Met	Met	Leu	Met
ATPase 6	226	8074	Val	Val	Val	Ile
COIII	261	9348	Ile	Val	Val	Val
ND3	114	9504	Thr	Thr	Thr	Ala
ND4L	97	9982	Val	Met	Met	Met
ND4	459	10426	Asn	Asn	Asn	Ser
		10431	Leu	Leu	Leu	Met
		10591	Asn	Asn	Asn	Ser
		10722	Ala	Ala	Ala	Thr
		10946	Ile	Met	Ile	Ile
		11211	Val	Val	Val	Ile
		11243	Met	Met	Met	Ile
		11394	Ile	Ile	Ile	Val
ND5	607	11772	Ile	Ile	Ile	Val
		11820	His	His	Tyr	Tyr
		11901	His	His	His	Tyr
		11922	Ile	Ile	Ile	Val
		11997	Leu	Leu	Leu	Met
		12042	Leu	Phe	Phe	Phe
		12238	Ala	Ala	Ala	Gly
		12549	Phe	Phe	Phe	Leu
		12829	Ile	Thr	Ile	Thr
		13135	Val	Val	Val	Ala
		13162	Ser	Ser	Ser	Asn
		13170	Val	Val	Val	Ile
		13281	Ala	Ala	Ala	Pro
		13438	Thr	Ile	Thr	Thr
ND6	172	13683	Val	Ile	Ile	Val
		13709	Gly	Gly	Asp	Gly
		13751	**Val**	**Val**	**Val**	**Ala**
		13755	**Cys**	**Cys**	**Cys**	**Tyr**
		13775-6	**Ile**	**Ala**	**Thr**	**Ile**
Cytb	381	14205	Ala	Thr	Ala	Ala
		14253	Val	Val	Val	Ile

[a] Base refers to the nucleotide numbering used by Bibb et al. (1981). Position 4706 was mislabeled 4702 by Hirama and Fischer Lindahl (1985). See Experimental Procedures for the sources of the mtDNA.
[b] Underlining marks the rarer forms of a given residue or the form different from that associated with α. Boldface marks positions where a peptide of suitable length for T cell recognition could be found in four allelic forms.
[c] The vertical lines mark residue substitutions within 50 nucleotides of each other which would correspond to a 17 amino acid long peptide.

Within the NADH dehydrogenase subunits ND2 and ND6 multiple changes occur that might alter one or more epitopes. Multiple substitutions occur around the middle of *ND6* within a span of 31 amino acids (Table 2). This span includes short sequences that fit the motif that Rothbard and Taylor (1988) have found in many T cell epitopes. The α, β, γ, and δ sequences all differ in the region spanning residues 96 to 104, with an epitopic motif at 94–98. However, the Mus spretus sequence also has a cluster of four substitutions in this span. Since the M. spretus form of MTF is indistinguishable from MTF^a (Fischer Lindahl et al., 1986), another sequence must be involved in the creation of the MTF antigen.

The most striking substitutions occur at base numbers 2766 and 2767 at the 5′ end of *ND1*, which encodes a 30 kd hydrophobic subunit of the NADH dehydrogenase complex. The resultant variation would generate a different amino acid for each of the four *Mtf* alleles at residue 6 of the protein (Table 3). Only a partial sequence exists for the mtDNA from M. spretus mice (Mtf^a); the predicted amino acid sequence matches the α sequence to residue 10. The leucine at residue 11 may not affect a T cell epitope centered around residue 6. The first 23 residues of ND1 are hydrophobic, followed by the charged Glu-Arg-Lys sequence. The pattern of 23 hydrophobic amino acids followed by these three charged residues is conserved in ND1 from other mammals. The first three residues of the rodent sequences are bracketed in Table 3 because GUG at position 1 is not an obvious initiation codon (see below).

Sequences Selected for Peptide Synthesis

To test the hypothesis that MTF equals the N-terminal peptide of ND1, we sought to show that targets, which normally are not lysed because they lack the appropriate *Mtf* allele, can be specifically recognized by CTLs following incubation with the correct peptide. We chose a C-terminus including the three charged residues at positions 24–26 to aid solubility, or ending at position 17, assuming that

Table 2. Amino Acid Sequences Surrounding a Polymorphic Site in ND6

	90	100	110	120	130	140
α	—— I L G F L V L **G V I M E V F L I C V** L N Y Y D E V G V I N L D G L G **D W L M Y** E V D D V G V **M L E** G G I G V ——
β	. . . . . A . . . . . I . . . . .
γ	. . . . . T . . . . . D . . . . . I . . . . .
δ	. . . . . Y A . . . . .
α(spretus)	. . . . . V V V Y . . . . . I . . V . . . A . . . . .

Boldface marks T cell epitope motifs (Rothbard and Taylor, 1988).

this was far enough from residue 6 to contain the T cell epitope. In mammalian mitochondria any AUN codon may be used for initiation (Bibb et al., 1981; Fearnley and Walker, 1987), and an AUU codon does occur at position 4. However, the published human and bovine *ND1* sequences begin at position 1 (Anderson et al., 1981, 1982), where the rodent sequences carry a GUG codon. Therefore, peptides were synthesized from both positions 1 and 4: three of α-type (residues 4–26, 1–26, and 1–17) and one of β-type (residues 1–17) (Table 4).

Nomenclature of Mouse Strains, Cell Lines, and CTLs

To clearly describe the specificity of our CTLs, we have adopted the following symbolism: C3H/HeJ [α,a;k] for example, describes the C3H/HeJ strain, which has the Mtf^{α} Hmt^{a} $H\text{-}2^{k}$ genotype. For CTLs, β-anti-α describes killer cells obtained after immunization of an Mtf^{β} Hmt^{a} mouse with cells from an *H-2*-matched Mtf^{α} Hmt^{a} mouse. Such effectors were specific for Mtf^{α} Hmt^{a} target cells, irrespective of their *H-2* genotype, demonstrating that classical H-2-restricted recognition does not apply to Mta. The rat–mouse somatic cell hybrids are similarly designated, thus 7E8 cells [ρ,a;b] expressed the rat Mtf^{ρ} (Loveland et al., unpublished data) and mouse Hmt^{a} and $H\text{-}2^{b}$ genes.

ND1α Peptides Replace the MTF$^{\alpha}$ Antigen

Figure 1 shows typical experiments to test whether peptides can mimic the MTF antigen. A β-anti-α CTL line lysed Mtf^{α} target cells and not those that expressed Mtf^{β} (lymphoblasts in Figure 1A and the F10.2 hybrid cells in Figure 1B). However, overnight incubation with either ND1α1-26 or ND1α1-17 peptide transformed F10.2 cells into highly susceptible targets, equivalent to normal lymphoblasts expressing Mtf^{α} (Figure 1B). By contrast, ND1α4-26 and ND1β1-17 peptides failed to generate α-target cells, and no lysis was obtained. A hybrid target, 7E8 [ρ,a;b], which has rat instead of mouse mtDNA and expresses the rat antigen MTF$^{\rho}$, shows occasional cross-reactive lysis by β-anti-α CTLs. 7E8 cells were strongly lysed after exposure to ND1α1-26 or ND1α1-17 (Figure 1C). The effect of the peptides was thus specific and dependent on both their amino acid sequence and N-terminal residues. ND1α4-26, with fMet at position 4 and lacking residues 1–3, is not recognized by anti-MTF$^{\alpha}$ CTLs, being either too short or of the wrong sequence. As MTF$^{\alpha}$-specific CTLs recognized the ND1α1-17 peptide but not ND1β1-17, which differs only at position 6, it is this residue that defines the allelic difference.

CTLs raised in Mtf^{δ} mice against Mtf^{α} cells could potentially recognize more and different antigens than do β-an-

Table 3. Amino-Terminal Sequences of ND1

	1 5 10 15 20 25
Mouse	
α and α(ddY)	(fM F F) I N I L T L L V P I L I A M A F L T L V E^{-}R^{+}K^{+} I . . .
α(spretus)	(- - -) - - - - - - - L . . .
β	(- - -) - - A - - - - - - - - - - - - - - - - - - - . . .
γ	(- - -) - - V - - - - - - - - - - - - - - - - - - - . . .
δ	(- - -) - - T - - - - - - - - - - - - - - - - - - - . . .
Rat	(- Y -) - - - - - - - I - - - - - G L - - - - - - - - - . . .
Hamster	(- H L) - - - - M - - I - - - - - - - . . .
Cow	- - M - - - - M - I I - - - L - V - - - - - - - - - V . . .
Human	- P M A - L - L - I - - - - - - - - - - M - T - - - - - . . .

Amino acid sequences are inferred from nucleotide sequences: mouse α (Bibb et al., 1981), β and γ (Yonekawa et al., unpublished data), δ, α(ddY), and α(spretus) (Wang et al., unpublished data), rat (Saccone et al., 1981), hamster (Kotin and Dubin, 1984), cow (Anderson et al., 1982), and human (Anderson et al., 1981).

Table 4. Synthetic ND1 Peptides

Peptide	1 . . . 5 . . . . 10 . . . . 15 . . . . 20 . . . . 25 .
ND1α1-26	fMFFINILTLLVPILIA[a]MAFLTLVERK
ND1α4-26	fMNILTLLVPILIA[a]MAFLTLVERK
ND1α1-17	fMFFINILTLLVPILIA[a]M
ND1β1-17	fMFFINALTLLVPILIA[a]M

[a] Position of [^{3}H]alanine.

ti-α CTLs, as the entire δ mitochondrial sequence differs from the α sequence at more positions than does β (Table 1). F10.2 and 7E8 target cells were recognized by δ-anti-α CTLs after incubation with ND1α peptides (Figures 1E and 1F, respectively) and lysed at least as well as *Mtf*$^{\alpha}$ targets (Figure 1D). Unlike the β-anti-α CTLs (Figure 1C), the δ-anti-α CTLs did not cross-react with the rat MTF antigen (Figure 1F), suggesting some difference in fine specificity. On this evidence the antigens recognized by the two types of CTLs were closely similar, if not identical, and were encoded by the *ND1* gene.

Competition Analysis Shows Exclusive Recognition of ND1 Peptides

The issue of whether the ND1 peptide is the sole determinant of the Mta antigen can be decided by inhibition assays. The lysis of labeled target cells can be completely inhibited only by unlabeled competitors carrying identical antigens; if the competitors express merely a subset of the antigens recognized by the CTLs, they will inhibit lysis incompletely, even at the highest doses. We used CTLs freshly derived from immunized mice, because they should have the broadest repertoire of antigen recognition. As expected, lysis of labeled C3H [α,a;k] targets by β-anti-α CTLs was efficiently reduced by C3H competitors, irrespective of their treatment with peptides (Figure 2A). Hybrid cells alone did not compete (Figures 2B and 2C), but after incubation with the ND1α1-26 or ND1α1-17 peptide, the F10.3 hybrid [β,a;q] cells were strong inhibitors of lysis (Figure 2B), and the ND1α1-17 peptide rendered 7E8 hybrid cells [ρ,a;b] competitive (Figure 2C). The degree of this inhibition, particularly when compared with competitors that had the α-type mitochondrial genome and the full array of potential antigens (C3H), proves that the ND1 peptides displayed all features of the MTF antigen and that there was no subpopulation of CTLs recognizing additional specificities. Control cells coated with the ND1β1-17 peptide were uniformly ineffective competitors, demonstrating the strict discrimination by the CTLs.

The ND1β1-17 Peptide Replaces the MTF$^{\beta}$ Antigen

The allelic specificity of the ND1β peptides is shown in Figures 3A and 3C. Two α-anti-β CTLs lysed the Bom101 T cell line from BOM [β,a;q] mice, but not C3H [α,a;k] target cells. Incubation of the C3H lymphoblasts with 400 nM ND1β1-17 transformed them into very sensitive targets. By contrast, ND1α1-17 proved ineffective in creating a lysable target for these CTLs.

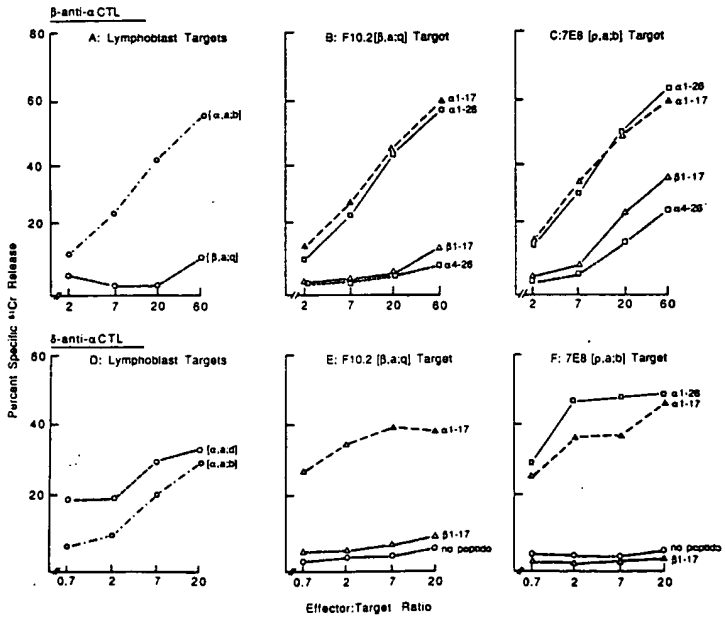

Figure 1. Recognition of the ND1α1-26 and ND1α1-17 but Not the ND1α4-26 or ND1β1-17 Peptides by β-Anti-α and δ-Anti-α CTLs

The Mtf-specific CTLs used were β-anti-α in (A), (B), and (C) and δ-anti-α in (D), (E), and (F). Target cells were lymphoblasts stimulated with concanavalin A, C3H.SW [α,a;b], and BOM [β,a;q] in (A) and B10.D2 [α,a;d] and BALB.B [α,a;b]) in (D); the F10.2 hybrid [β,a;q] in (B) and (E), and the 7E8 hybrid [ρ,a;b] in (C) and (F). The target cells in (B), (C), (E), and (F) were preincubated for 20 hr alone (open circles) or with the peptides ND1α1-26 (closed squares), ND1α1-17 (closed triangles), ND1α4-26 (open squares), and ND1β1-17 (open triangles) at 400 nM before labeling with ^{51}Cr.

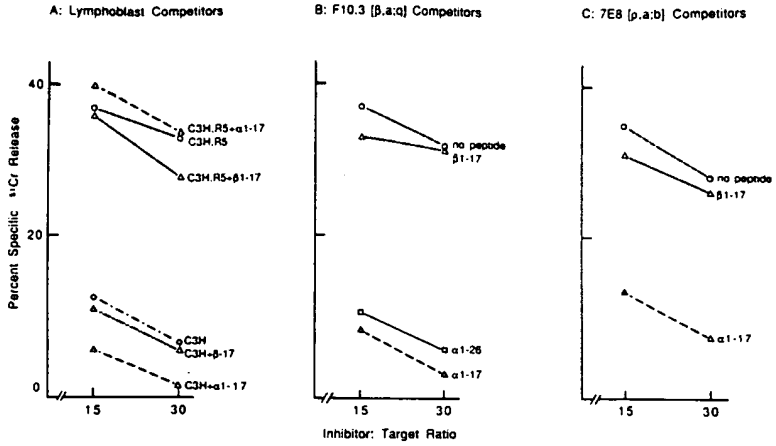

Figure 2. Inhibition of Lysis of *Mtf*$^{\alpha}$ Targets by Peptide-Treated Cells in an Allele-Specific Manner and Dependent on the Expression of *Hmt*^{a}

Lysis of C3H [α,a;k] ^{51}Cr-labeled target cells by β-anti-α CTLs (E:T = 7:1) was assayed with inhibitor to target (I:T) ratios of 15:1 or 30:1. The range of target cell lysis in the absence of competitors is shown by the shaded area. Competitors were C3H [α,a;k] or C3H.R5 [α,b;k-c4] lymphoblasts in (A), the F10.3 [β,a;q] hybrid in (B), and the 7E8 [p,a;b] hybrid in (C), each preincubated alone or with 400 nM peptides for 16 hr.

Competition assays confirmed the specificity of α-anti-β CTLs and their recognition of the ND1β1-17 peptide as the equivalent of MTF$^{\beta}$. In Figure 3B the only inhibiting targets were F10.3 cells [β,a;q] and the C3H cells after treatment with ND1β1-17. Target cells coated with this peptide were more effective than B6. *Mtf*$^{\beta}$ [β,a;b] and BOM [β,a;q] lymphoblast competitors (Figure 3D). ND1α1-17 peptide was not recognized as antigen in either experiment.

Recognition of ND1 Peptides Depends on *Hmt*

MTF is recognized only on cells that express the *Hmt* gene product, which, we have reason to believe, is an MHC class I molecule. *Hmt*^{a} or *Hmt*^{c} mice express cross-reacting forms of Mta, whereas the few strains that carry the *Hmt*^{b} allele do not express Mta in a form so far recognizable. Cells of one such strain, C3H.CAS(R5) [α,b;k-c4], did not compete with *Mtf*$^{\alpha}$ targets for β-anti-α CTLs, whereas cells of its congenic control, C3H, competed efficiently (Figure 2A). Peptide treatment of C3H.CAS(R5) cells had no effect on their phenotype (Figure 2A).

The α-anti-β CTLs also failed to lyse the C3H.CAS(R5) *Hmt*^{b} cells, and peptide treatment was ineffective (Figures 3A and 3B). Cells from C3H.CAS(R4) [α,b;k], another *Hmt*^{b} strain congenic with C3H, were equally inefficient competitors of α-anti-β CTLs, whether treated with the ND1β1-17 or the ND1α1-17 peptide (Figure 3D). The weak inhibition sometimes seen with these *Hmt*^{b} cells (Figures 3B and 3D) has no obvious immunological basis, as in no experiment were R4 or R5 cells themselves lysed, whether incubated with peptide or not.

Target Cell Lysis Depends on Peptide Concentration

Incubation with peptide for more than 3 hr was necessary for efficient generation of new target antigens. The three peptides that were recognized on appropriate target cells were effective at similar concentrations. Overnight incubation with 100 nM peptide produced maximal or near maximal target cell lysis in each case, the effect falling away at lower concentrations and disappearing at less than 10 nM (Figure 4). The minimal lysis of 7E8 cells in the absence of peptide was due to the known weak cross-reactivity of anti-α CTLs with the rat MTF^{p} antigen (Figure 4A).

While we state the molar concentration of added peptide, the effective local concentration might have been quite different: higher if the cells took up peptide in aggregated form, and lower if only the soluble fraction were functional. The hydrophobic peptides were suspended in dimethyl sulfoxide (DMSO). Their solubility could be estimated by determining the [^{3}H]alanine in the supernate after centrifuging the peptide stock preparations. The results (ND1α1-26 16% soluble, ND1α1-17 4% soluble, and ND1β1-17 ~2% soluble) suggested that the uptake of these hydrophobic peptides by cells and their expression as antigen included the particulate fraction. If only the soluble fractions had been involved, substantial differences in the titration curves for ND1α1-26 and ND1β1-17 should have been found. In fact, when the particulate fraction of ND1α1-17 was removed before incubation with target cells, thus changing the nominal concentration from 100 to 4 nM, no lysis was obtained. It follows also that the lack of recognition of the ND1α4-26 peptide (6% soluble) was not due to its insolubility. DMSO did not affect antigenicity: killing depended solely on the CTL specificity and the peptide sequence, and treatment with the "wrong" peptide or with DMSO itself did not change the phenotype of target cells.

A Variety of Cell Types Present ND1 Peptides

CTLs normally recognize antigens synthesized inside the target cell, whether the information came from the cellular or infecting viral genomes. For antigens added in the medium, it is not clear by what path(s) they become associated with class I molecules. The hydrophobic ND1 peptides were tested on several cell types and functioned as antigen on all of them, either by creating new target epitopes or, in some cases, causing enhanced lysis (Table 5).

L929 [α,a;k] fibroblast targets, whether untreated or incubated with ND1α1-17, were lysed to a similar degree by anti-MTF$^{\alpha}$ CTLs; they became targets of α-anti-β CTLs only after treatment with ND1β1-17. Lymphoid cell lines such as the Bom101 T cell or the rat–mouse T cell hybrids (7E8, F10.2, and F10.3) behaved similarly: they were sensitive conventional target cells and expressed new MTF antigens following incubation with the appropriate peptide.

The P1.HTR mastocytoma, a mutant of the P815 cell line (Van Pel et al., 1985), expresses *Mtf*$^{\alpha}$; after treatment with

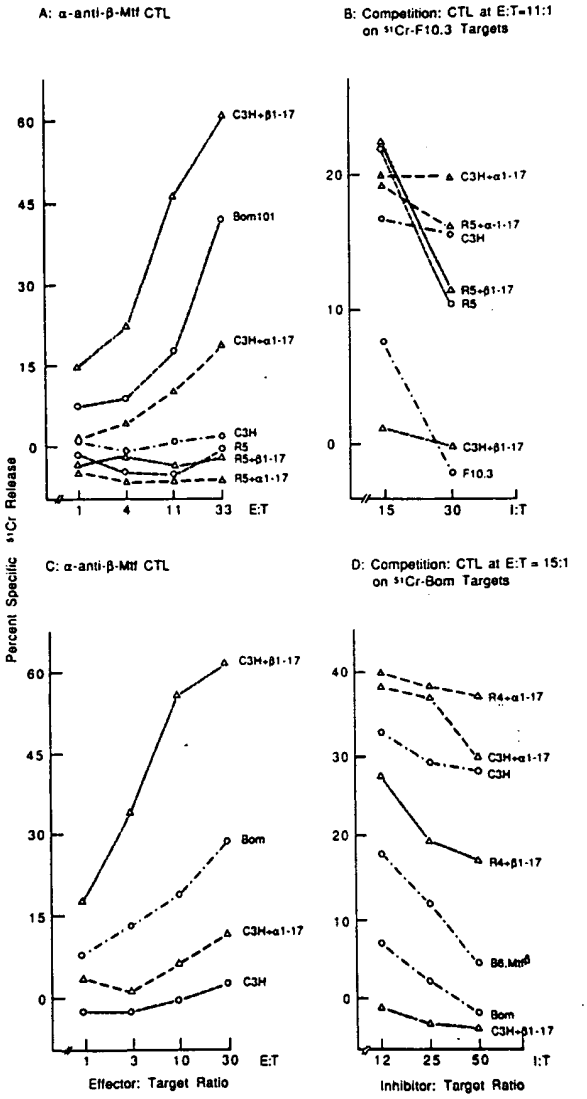

Figure 3. α-Anti-β CTLs Recognize Target Cells Treated with ND1β1-17 but Not ND1α1-17 Peptide, Dependent on the Expression of *Hmt*[a]

Test target cells were ^{51}Cr-labeled ([A] and [C]) or used as competitors ([B] and [D]). One α-anti-β CTL culture was used in (A) and (B), and a different one was used in (C) and (D). The range of lysis in the absence of inhibitors is shown by the shaded area. Target cells were preincubated alone or with 400 nM peptide overnight. Abbreviations are R5 for C3H.CAS(R5) [α,b;k-c4] cells and R4 for C3H.CAS(R4) [α,b;k] cells.

a high concentration (1.5 μM) of the homologous ND1α1-26 peptide, lysis was enhanced. Lower concentrations, such as 250 nM, used in other experiments did not have this effect.

Peritoneal exudate cells, mostly macrophages and monocytes with few lymphocytes, are relatively resistant to Mta-specific lysis. Incubation of these cells with ND1α1-26 peptide overnight converted them into sensitive targets, their enhanced lysis due in all probability to an increase in cell surface peptide antigen. Peritoneal exudate cells from BALB.B [α,a;b] or SHGBF1 mice [δ,a;g/b], poor targets originally, were extensively lysed after peptide treatment. Fresh spleen cells are moderately sensitive targets, and lysis was greatly enhanced after peptide treatment. No such enhancement was observed with the more homogeneous and CTL-sensitive populations of concanavalin A–stimulated spleen or lymph node cells, which were used in the figures. The common feature of several cell types—that weak lysis could be increased by adding the homologous antigen in peptide form—suggests that in vivo MTF might be limiting for CTL recognition.

Discussion

In this paper we have identified the minor H antigen MTF as a hydrophobic peptide, derived from a known mitochondrial protein, ND1. Substitutions in a single codon of the *ND1* gene, corresponding to position 6 of the protein, created the *Mtf* alleles. MTF$^{\alpha}$ has an isoleucine at this position, β has alanine, γ has valine, and δ has threonine. The flanking sequences are identical. Both β-anti-α and δ-anti-α CTLs recognized cells treated with the ND1α1-17 and ND1α1-26 peptides. The fact that the degree of recognition matched that of target cells expressing endogenous MTF, also in competitive assays, implies that the same determinants were involved in both and that they were completely described by the peptides. The similar recognition of ND1β1-17 by α-anti-β CTLs strengthens this conclusion. We expect that synthetic peptides can also substitute for the MTF$^{\gamma}$ and MTF$^{\delta}$ antigens. Hmt-restricted recognition of the mitochondrial MTF peptide is analogous to H-2-restricted recognition of viral peptides in infected cells.

While it is clear that only the N-terminal peptide of ND1 contributes to Mta, other mitochondrial polymorphisms, such as in ND6, might be recognized as minor H antigens in a standard H-2-restricted fashion. In principle, additional mitochondrial antigens could arise from alternate reading frames in the mitochondrial genome or by transcription from the D-loop, generating exportable protein sequences. The efficient inhibition of target cell lysis by synthetic ND1 peptides suggests that if any of these hypothetical processes do occur, they do not create antigens recognized by Hmt-restricted CTLs.

It was informative that the peptides beginning at position 1 (codon GUG) were antigenic, whereas the one synthesized from position 4 (codon AUU) was not. In mitochondria, an AUN codon is generally the site of translation initiation (Anderson et al., 1981; Montoya et al., 1981; Fearnley and Walker, 1987). However, our data show that mouse ND1 begins at position 1, as do the sequences identified for *ND1* (*URF1*) of human and bovine mtDNA (Anderson et al., 1981, 1982). It has been proposed, purely on the basis of sequence comparisons, that rodent (mouse, rat, and hamster) *ND1* genes are translated starting with the GUG codon, which is occasionally used for initiation in diverse eukaryotic and prokaryotic genes (Kotin and Dubin, 1984; Gadaleta et al., 1988). Alternative initiation codons are used by prokaryotes, and a CUG is proposed as the normal initiation codon of the human *c-myc* gene (Hann et al., 1988). It is conceivable that translation begins from both sites of the mouse *ND1* gene, and that only the protein starting at position 1 is processed to yield MTF.

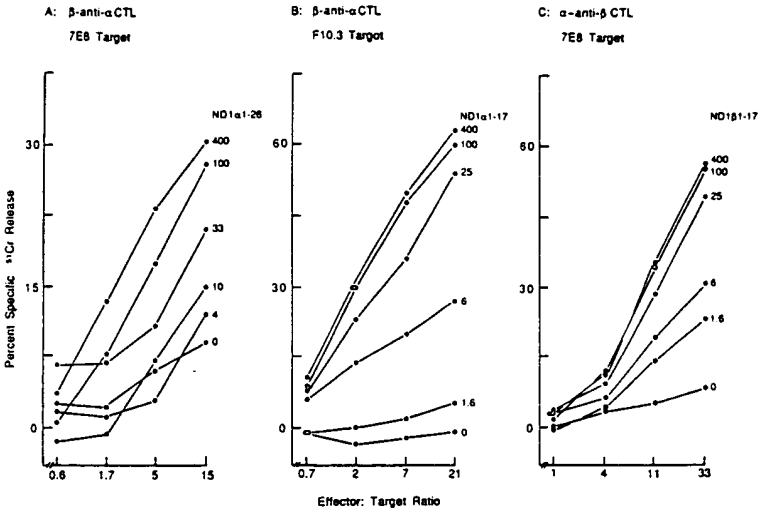

Figure 4. Titration of Peptides Demonstrates Does-Dependent Target Cell Lysis

The 7E8 [ρ,a;b] and the F10.3 [β,a;q] hybrid target cells were preincubated for 18 hr with or without peptide at the concentrations shown (nM).

The N-terminal stretch of *ND1* is extremely hydrophobic with strong sequence conservation among such diverse organisms as Drosophila, Xenopus, locust, and sea urchin, several clusters of residues being identical (Garesse, 1988; Roe et al., 1985; McCracken et al., 1987; Cantatore et al., 1988). In 14 of 15 species for which proteins sequences can be deduced, the sequence Glu-Arg-Lys (mammalian residues 24–26 in Table 3) follows a stretch of at least 18 noncharged residues. This points to a conserved function, perhaps the correct insertion and orientation of the ND1 subunit in the mitochondrial inner membrane.

There are no precedents to suggest how a mitochondrial peptide might reach the cell surface to be recognized by T cells, and the physiological significance of this event is unclear. Position 18 of ND1, following Ala-Met-Ala, can be identified as a possible cleavage site for signal peptidases (von Heijne, 1986). However, it is unlikely that MTF is generated as a cleaved leader sequence: the mature ND1 proteins of the cow (Yagi and Hatefi, 1988) and of Neurospora crassa (Zauner et al., 1985) have the intact N-terminal residues predicted from the gene sequence. There is no reason to assume that it is otherwise in the mouse. Rather, MTF might arise from the natural autophagic recycling of the cell's constituents, including the mitochondria. This would imply a significant precursor pool of MTF with a long half-life.

Nonlysosomal degradation in the cytosol (Bigelow et al., 1981), close to or within the endoplasmic reticulum, may be the means of removing abnormal or excess subunits of newly synthesized protein complexes (Lippincott-Schwartz et al., 1988). Amino acid misincorporation and premature termination also occur at a sufficiently high frequency to generate many potential antigenic peptides (Kourilsky and Claverie, 1989). ND1 is one of many subunits in the NADH dehydrogenase complex, and the mitochondria may have mechanisms to balance the amounts of imported and locally made subunits, to dispose of excess, and to remove incomplete proteins. Accordingly, MTF could be an immediate byproduct of the translation of *ND1*

Table 5. Cell Types on Which ND1 Peptide Was Recognized and Examples of Enhanced Target Lysis

Target Cell	Genotype	CTL	E:T	% Specific ^{51}Cr Release		Result[b]
				Without Peptide	With Peptide (nM, ND1)[a]	
L929 fibroblast	[α,a;k]	β-anti-α	7:1	23	26 (400, α1-17)	N
L929 fibroblast	[α,a;k]	α-anti-β	11:1	3	19 (400, β1-17)	A
7E8 T cell hybrid	[ρ,a;b]	β-anti-α	7:1	13	36 (400, α1-17)	A
F10.3 T cell hybrid	[β,a;q]	β-anti-α	7:1	−2	39 (400, α1-17)	A
Bom101 T cell line	[β,a;q]	β-anti-α	7:1	−1	18 (400, α1-17)	A
C57BL/6 splenocytes	[α,a;b]	β-anti-α	10:1	22	35 (250, α1-26)	E
SHGBF1 splenocytes	[δ,a;g/b]	β-anti-α	10:1	−1	34 (250, α1-26)	A
SHGBF1 PEC[c]	[δ,a;g/b]	β-anti-α	7:1	1	50 (720, α1-26)	A
BALB.B PEC	[α,a;b]	β-anti-α	7:1	19	55 (720, α1-26)	E
P1.HTR mastocytoma	[α,a;d]	β-anti-α	12:1	33	50 (1500, α1-26)	E
P1.HTR mastocytoma	[α,a;d]	β-anti-α	14:1	61	55 (250, α1-26)	N

[a] Target cells were incubated overnight with the concentrations of the peptides listed, labeled with ^{51}Cr, and added to CTLs at the effector to target (E:T) ratios shown. The data are collated from five experiments.

[b] Incubation with peptide either caused enhanced lysis (E) of target cells, addition of an MTF antigen (A) enabling recognition, or had no effect (N) on target cell lysis.

[c] Peritoneal exudate cells were obtained from normal mice.

mRNA; if so, MTF would be rapidly affected by inhibition of mitochondrial protein synthesis (Han et al., 1989).

Boon and Van Pel (1989) have speculated that small subgenic fragments of DNA (peptons) are transcribed systematically and translated into peptides that become subject to immune surveillance via the MHC class I presentation system. Such a system could generate a diversity of minor H antigens without flooding the class I molecules by peptides derived from the most abundant cellular proteins. However, this model fails to explain why all peptides defined to date, including MTF, are read in the standard frame, or why deletion of the promoter and first exon of the P91A gene should affect the expression of an epitope encoded in the fourth exon (Lurquin et al., 1989).

Several attempts have been made to define general characteristics of T cell epitopes (DeLisi and Berzofsky, 1985; Spouge et al., 1987; Bastin et al., 1987; Rothbard and Taylor, 1988). DeLisi's model of amphipathic structure relates to antigens recognized by class II–restricted $CD4^+$ T cells rather than to class I–restricted $CD8^+$ T cells. Rothbard's motif requires a charged group or glycine followed by hydrophobic residues and another charged or polar group (see Table 2). The ND1 N-terminus has none of these patterns, being entirely without charged residues. We have no indication that the ND1 peptides can bind to the usual restricting molecules, H-2K, D, and L. Hmt thus appears as an exceptional class I molecule, binding peptides that differ in their chemical properties from those displayed by other MHC molecules.

The uptake of ND1 peptides differs from that of other peptides defining T cell epitopes. For example, the easily soluble peptides of the influenza nucleoprotein were rapidly taken up by target cells and recognized by CTLs (Townsend et al., 1986, 1988). The much slower expression of ND1 peptides is perhaps not surprising in view of their hydrophobicity. More interesting is the possibility that Hmt might be charged with peptide by a different mechanism or might sample intracellular peptides from a different compartment than do the other MHC class I molecules (Braciale et al., 1987; Benacerraf, 1988; Townsend et al., 1989). Unlike some peptides bound by H-2K (Hosken et al., 1989), the ND1 peptides do not become associated with Hmt at the cell surface: preliminary experiments show that pinocytosis, induced by treatment with hypertonic buffer, shortens the lag before peptide recognition.

The recently cloned tumor transplantation antigens, P91A, P35B, and P198, are quite similar to MTF, although the proteins remain to be identified. Each arose by mutation in a different nuclear gene, causing a single conservative amino acid substitution, and each can be represented by a synthetic peptide (De Plaen et al., 1988; Boon et al., 1990).

Mta, the unusual minor H antigen, became the proverbial exception to prove the rule that minor H antigens are processed peptides that bind to the MHC class I or class II restriction molecules. A conservative substitution, which does not affect the function of the protein, may enable a processed peptide to bind to an MHC molecule for presentation to T cells involved in immune surveillance, as in the case of the P91A tumor antigen (Lurquin et al., 1989), or it may change the epitope of an already binding peptide, as in the case of MTF. Thus, minor H antigens can arise by mutation in any protein, regardless of its function or cellular location.

Experimental Procedures

Sequencing Mitochondrial DNA

Fragments of mtDNA were cloned into the M13 vectors, mp18 and mp19, and sequenced by the Sanger dideoxy method (Sanger et al., 1977). When inserts were longer than 1 kb, custom oligonucleotides were synthesized and used as sequencing primers. The β-type mitochondrial genome was from the NZB/BINJ strain, γ-type from descendants of a wild female mouse caught near Pavia, Italy, and δ-type from the SUB-SHH stock derived from wild mice caught near Shanghai, China (Fischer Lindahl, 1986). The α(ddY) genome was derived from the Japanese ddY strain (Yonekawa et al., 1982) and maintained in the congenic C57BL/10Sn-mtJ strain (Hayashi et al., 1987); the α(spretus) genome was from a Moroccan M. spretus, maintained in the congenic B6.mt^{Spr} strain (Fischer Lindahl et al., 1986).

Mice

The *Mtf*, *Hmt*, and *H-2* type of each mouse strains is given in brackets as described in the Results. The standard inbred strains C57BL/6J [α,a;b], C3H/HeJ [α,a;k], C3H.SW/SnJ [α,a;b], BALB/cJ [α,a;d], NZW/LacJ [α,a;z], (NZB/BINJ×NZW/LacJ)F1 [β,a;d/z] and B10.D2/nSnJ [α,a;d] were obtained from Jackson Laboratories, Bar Harbor, Maine. BALB.B/Ola [α,a;b] mice were purchased from Olac Ltd., Bicester, Oxon, England. NMRI/Lac [α,a;q] (Ferris et al., 1983a), B6.Mtf^{β} [β,a;b] (Fischer Lindahl et al., 1986), and the following strains were bred in our colony. BOM [β,a;q] mice were derived by brother–sister mating for six or more generations from the outbred NMRI/Bom stock (Ferris et al., 1983a). C3H.CAS(R4) [α,b;k] is a congenic recombinant strain between C3H/HeJ and an $H\text{-}2^{cas3}$ strain with $H\text{-}2K^k$ D^k Hmt^b (Richards et al., 1989). C3H.CAS(R5) [α,b;k-c4] is a congenic recombinant strain between C3H/HeJ and an $H\text{-}2^{cas4}$ strain with $H\text{-}2K^k$ A^{c4} D^{c4} Hmt^b (Steinmetz et al., 1986). BALB.SHC [δ,a;d] and SHGBF1 [δ,a;g/b] (a heterozygote of BALB.SHG [δ,a;g] and BALB.B [α,a;b]) have been derived by backcrossing SUB-SHH females (see above) to BALB/c, BALB.G, or BALB.B *H-2* congenic males.

Cell Lines and Targets

The 7E8 [ρ,a;b], F10.2, and F10.3 [β,a;q] somatic cell hybrids which carry rat or mouse mtDNA (thus Mtf^{ρ} or Mtf^{β}) were produced by fusion of C58(NT)D rat thymoma cells with concanavalin A–stimulated Mtf^{β} mouse lymphoblasts, followed by selection in HAT medium (Loveland et al., unpublished data). Bom101 is a noncytotoxic T cell line derived from a BOM [β,a;q] mouse. P1.HTR, a mutant subline of the DBA/2 strain P815 mastocytoma, was a gift from Dr. Thierry Boon (Van Pel et al., 1985). L929 fibroblasts (of C3H [α,a;k] origin) were from laboratory stocks. The cell lines were cultured in RPMI-1640 supplemented with 5%–10% fetal calf serum, 50 μM 2-mercaptoethanol, L-glutamine, penicillin, and streptomycin. Lymphoblast targets were generated from fresh spleen or lymph node cell suspensions by incubation with concanavalin A at 2–4 μg/ml for 1 to 4 days. Peritoneal exudate cells were obtained by flushing the peritoneum of normal donor mice with cold phosphate-buffered saline.

Cytotoxicity Assays

The *Mta* phenotype of target cells was determined by a standard chromium release assay (Fischer Lindahl et al., 1986). In brief, cytotoxic effector T cells were generated in a 5 to 8 day mixed lymphocyte culture, using 10^7 responder spleen cells from immunized mice mixed with 10^7 irradiated (3500 Rad) stimulator spleen cells per 4.5 cm^2, 3 ml tissue culture well. The immunizing combinations, always using *H-2*-matched strains, were BOM [β,a;q] anti-NMRI/Lac [α,a;q] or B6.Mtf^{β} [β,a;b] anti-C57BL/6J [α,a;b] to make β-anti-α CTLs, and the reverse combinations to generate α-anti-β CTLs. (NZB/BINJ×NZW/LacJ)F1 [α,a;d/z] anti-NZW/LacJ [α,a;z] was also used for β-anti-α CTLs. For δ-anti-α CTLs the BALB.SHC [δ,a;d] anti-BALB/c [α,a;d] combination was used. Responder mice were given three intraperitoneal injections

of 10^7 antigenic spleen cells at 3 week intervals, and their spleen cells were used between 1 and 20 months later. In the assay, effector cells (E) were titrated against 10^4 targets (T) labeled with ^{51}Cr as sodium chromate, using E:T ratios in the range 0.5:1 to 60:1, as cell numbers permitted. Normally, four 3-fold dilutions in 0.2 ml were tested in triplicate. Following a 3.5 to 4 hr incubation at 37°C, 0.1 ml samples of the supernatants were harvested, and the ^{51}Cr released from the target cells was measured in a gamma counter. The percent specific ^{51}Cr release was calculated as 100 × (test release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release varied with the target cells used from <5% to 30%.

In competition assays, 10^4 ^{51}Cr-labeled target cells were mixed in duplicate with unlabeled inhibitor (I) cells to yield 2-fold titration steps from I:T = 60:1 to 15:1 or 50:1 to 12.5:1. After addition of a constant number of effector cells (normally $\geq 10^5$), incubation, sampling, and analysis was the same as above.

Peptide Synthesis and Use

Peptides were synthesized on an Applied Biosystems Model 430A peptide synthesizer, using t-Boc amino acids coupled under standard manufacturer's conditions. One residue of [^{3}H]alanine was incorporated into each peptide to enable radioactive tracing, and N-formyl-L-methionine (Chemical Dynamics Corporation #45-5130-00) was coupled to the N-terminus as a preformed HOBT ester using dicyclohexylcarbodiimide in N-methylpyrrolidone for activation. No modification of the N-formyl-L-methionine was necessary before coupling. The products were deprotected and cleaved with hydrofluoric acid, extracted into DMSO, in which they were partially soluble, and not lyophilized. Analytical sequencing of each preparation was performed to show that the correct sequence had been obtained. Stock concentrations in DMSO were estimated to be 72 μM ND1α1-26, 40 μM ND1α4-26, 300 μM ND1α1-17, and 177 μM ND1β1-17. The peptide suspensions in DMSO were diluted in Hanks' balanced salt solution (GIBCO #310-4060) and added to prospective target cells at concentrations <800 nM so that DMSO, at ≤1% (v/v), was not toxic. The cells were cultured overnight at 37°C in RPMI-1640 containing 10% fetal calf serum, before labeling as required with ^{51}Cr.

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